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(57) Abstract

This invention relates to drug binding proteins, to genes encoding same and to assays and methods for screening pharmaceuticals. More specifically, this invention relates to a Cytokine Suppressive Anti-Inflammatory Drug (CSAID) binding protein, to a gene encoding same and to assays and screens useful in the evaluation and characterization of drugs of this pharmacologic class.

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DRUG BINDING PROTEIN

Field of the Invention:

This invention relates to drug binding proteins, to genes encoding same and to assays and methods for screening pharmaceuticals. More specifically, this invention relates to Cytokine Suppressive Anti-Inflammatory Drug (CSAID) binding proteins, to genes encoding same and to assays and screens useful in the evaluation and characterization of drugs of this pharmacologic class.

Background of the Invention:

Cytokines play an important role in regulating the cellular response during inflammation and other immune functions. Of particular interest are the cytokines interleukin-1 (IL-1, α and β) and tumor necrosis factor (TNF, α and β), which are the intercellular proteins involved in the initial step of the inflammatory response cascade (Arai, et al., Ann. Rev. Biochem. 59: 783-836 (1990)). Thus, there has been a substantial amount of research recently devoted to interfering with the production of IL-1 and TNF in response to an inflammatory stimulus.

One therapeutic approach involves suppressing the production of IL-1 and TNF at the level of transcription and/or translation and/or secretion. The activities associated with certain of pyridinyl imidazoles led to a class of compounds referred to as "CSAIDs", or Cytokine Suppressing Anti-Inflammatory Drugs (Figure 1). These compounds appear to arrest the expression of IL-1 and TNF predominantly at the translational level, although a lesser effect on transcription has also been observed but effects on other steps cannot be ruled out.

The pyridinyl imidazole, 5-(4-pyridyl)-6(4-fluorophenyl)-2,3-dihydroimidazo(2,1-b)thiazole (SK&F 86002) was identified as the prototypic CSAID. The basis for its activity has been established and characterized (Lee, et al., Int'l. J. Immunopharm. 10(7): 835-843 (1988); Agents and Actions 27(3/4): 277-279 (1989) and Int'l. J. Immunother. 6(1):1-12 (1990)). SAR studies (discussed herein) suggest that cytokine suppressive effect of the pyridinyl imidazoles represents a unique activity independent of their inhibitory effects on eicosanoid and leukotriene production. However, no compound of the initial series was selective for cytokine suppressive activity or was particularly potent.

Since the CSAIDs have substantial potential as novel anti-inflammatory therapeutic agents, there is significant interest in characterizing their mechanism of action at the molecular level, as well as btaining compounds with increased selectivity and potency. Specifically, identification and characterization of the CSAID molecular target would enhance the understanding of the biochemical processes involved in inflammation and aid in the design and screening of more potent anti-inflammatory drugs. This invention discloses, inter alia, the purification and characterization of such CSAID binding proteins (CSBPs).

The DNAs of this invention , such as the specific sequences disclosed herein, are useful in that they encode the genetic information required for the expression of the novel CSBPs. Additionally, the sequences may be used as probes in order to isolate and identify any additional members of the CSBP family as well as forming the basis of antisense therapy for disease conditions which are characterized by atypical expression of the CSBP gene. The novel protein itself is useful directly as a therapeutic or diagnostic agent as well as a component in a screening system for compounds which are antagonists or agonists of CSAID binding activity. The protein is also useful for eliciting antibody production in heterologous species, said antibodies being useful for the aforesaid diagnostic, therapeutic and screening applications. These and additional uses for the reagents described herein will become apparent to those of ordinary skill in the art upon reading this specification.

Brief Description of the Invention:

This invention provides isolated nucleic acid molecules encoding a CSAID binding protein, including mRNAs, DNAs, cDNAs as well as antisense analogs thereof and biologically active and diagnostically or therapeutically useful fragments thereof.

This invention also provides recombinant vectors, such as cloning and expression plasmids useful as reagents in the recombinant production of CSAID binding proteins or peptides, as well as recombinant prokaryotic and/or eukaryotic host cells comprising the CSBP encoding nucleic acid sequence.

This invention also provides methods of identifying ligands capable of binding to the CSBP by measuring the binding of the ligand to be identified relative to known ligands.

This invention also provides methods for screening drugs to identify compounds which interact with and bind to the CSBP. The binding protein may be in isolated form in solution, or in immobilized form, or may be genetically

engineered to be expressed n the surface of recombinant host cells such as in phage display system or as fusion proteins. Alternatively, whole cells or cytosolic fractions comprising the CSBP may be employed in screening protocols. Regardless of the form of the binding protein, a plurality of compounds are contacted with the binding protein under conditions sufficient to form a compound/ binding protein complex and compound capable of forming, enhancing or interfering with said complexes are detected.

This invention also provides nucleic acid probes comprising nucleic acid molecules of sufficient length to specifically hybridize to CSAID binding protein-like sequences.

This invention also provides an antisense oligonucleotide having a sequence capable of binding with mRNAs encoding the CSBP so as to prevent the translation of said mRNA.

This invention also provides transgenic non-human animals comprising or lacking a nucleic acid molecule encoding a CSBP. Also provided are methods for use of said transgenic animals as models for differential binding protein expression, mutation and SAR evaluation as well as in ligand and drug screens.

This invention also provides fusion proteins comprising a CSAID binding domain and a binding protein/ligand binding indicator domain capable of providing an analytically detectable signal. Also provided are methods of screening drugs by forming, enhancing or interfering with the detectable signal.

This invention also provides method of screening compounds to identify those compounds which bind to a CSAID binding protein comprising: providing a recombinant host cell expressing on the surface thereof a CSAID binding protein, said protein being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said protein; contacting a plurality of candidate compounds with said host cells under conditions sufficient to permit binding of compounds to the binding protein; and identifying those compounds capable of binding by detecting the signal produced by said second component.

Brief Description of the Figures

Figure 1 illustrates the correlation of IC_{50} of the pyridinyl imidazole CSAIDs for IL-1β biosynthesis in THP.1 cells and human monocytes. A Log-Log scatter plot of ~50 compounds with regard to their IC_{50}s for inhibiting IL-1 or TNF

was generated. Regression analysis was performed and the correlation coefficient is 0.881.

Figure 2 illustrates the time dependent and reversible uptake of ^{3}H-Compound I in intact THP.1 cells. 2 million THP.1 cells were incubated alone (appropriate solvent control) or with radiolabeled Compound I (50 nM) in the absence (0 - 0) or presence of excess non-radioactive ligand (50 μM) Compound I (square) and Compound VIII (triangle). At various intervals, the cells were centrifuged over a 8% sucrose cushion and the cell pellet was assessed for radioactivity by scintillation counting. Saturable binding was achieved at 15 minutes.

Figure 3 illustrates the subcellular localization of binding activity. 10 million THP.1 cells were incubated with 50 nM radiolabeled Compound I for 30 minutes at 22°C. The cells were disrupted by dounce homogenization. The cell lysate was fractionated into nuclear, particulate and soluble fraction by differential centrifugation. The bulk of radioactivity was associated with the cytosolic fraction. An identical result was obtained in a binding assay using previously fractionated samples.

Figure 4 illustrates the binding isotherm and Scatchard plot analysis of Compound I binding by THP.1 cytosol. Titration of radiolabeled Compound (0 to 1 μM) in the presence of constant excess cold ligand (50 μM) was performed in the binding assay using crude THP.1 cytosol. The specific binding is saturable. Scatchard plot analysis demonstrated a Kd of 3.6 nM, Bmax of 5 pmol/mg protein and a single site binding.

Figure 5 illustrates the specificity of the CSAID binding activity. A large number of the pyridinyl imidazole compounds spanning three different structural classes with known IC_{50}s for cytokine synthesis inhibition were tested in a competitive binding assay in which radiolabeled Compound I was used. There was a high degree of correlation between the two activities (R=0.889) suggesting that the binding event is a necessary step in the inhibition of cytokine production.

Figure 6 illustrates the regioselectivity of the CSAIDs. Four pairs of regioisomeric forms of the CSAIDs were tested in the bioassay and the competitive binding assay. Only one isomeric form of the respective pair was active with identical IC_{50}s in both assays.

Figure 7 illustrates that the binding of radiolabeled SB 202190 is saturable, specific and reversible. THP.1 cytosol was in cubated with 50 nM radiolabeled SB Compound I for 15 minutes to allow saturable binding to equilibrate, at which time

30 μM of the cold ligand was added and at various intervals, specific binding was determined. The binding is reversible with Compound VII and to a lesser extent, Compound XI and not at all with Compound VIII, the IC_{50}s of these compounds in the bioassay were 20 nM, 50 nM and >5 μM respectively.

Figure 8 illustrates the CSAID binding activity is protease and heat sensitive. THP.1 cytosol was subjected to trypsin (100 μg/ml) (Panel A) and heat (56°C)(Panel B) treatment. Maximum abrogation of binding activity was achieved within 2 minutes after treatment with trypsin. The binding activity was abrogated after incubation at 56°C, showed a gradual loss at 37°C and was relatively stable at 22°C and 4°C.

Figure 9 illustrates the analysis of photoaffinity labeling of CSBP by SDS-PAGE and Autoradiography. Approximately 40 μg of protein was pre-incubated with the inhibitors listed above the gel at 10 μM before photoaffinity labeling with ^{125}I Compound IV (2.5 nM). The reactions were analyzed by SDS-PAGE and autoradiography as described herein.

Figure 10 illustrates that analysis of fractions from preparative isoelectric focusing. Protein labeled with ^{125}I Compound IV was applied to the Rainin RF3 and analyzed as described herein.

Figure 11 illustrates the analysis of preparative SDS-PAGE fractions by (A) SDS-PAGE and Silver Staining, and (B) Radioactivity. Fractions were analyzed as described hereinbelow.

Figure 12 illustrates the homology of unique amino acid sequence discovered during analysis of CSBP to MAP kinase. The peptide sequence is listed below the linear representation of MAP kinase of the 15 residues; 9 identical (60%), 13 identical or homologous (87%).

Figure 13 illustrates the nucleic acid sequence and amino sequence of a portion of the CSAIDs Binding Protein.

Figure 14 illustrates the nucleic acid sequence of a second portion of the CSAIDs Binding Protein.

Figure 15 illustrates diagramatically the various CSBP cDNAs described herein.

Figure 16 illustrates the cDNA and amino acid sequence of one of the CSBP disclosed herein.

Figure 17 illustrates the difference in nucleotide and amino acid sequence between CSBP-1 and CSBP-2.

Figure 18 illustrates a phylogenetic tree of various protein kinases.

Figure 19 illustrates the alignment of the amino acid sequences of CSBP-1 and CSBP-2 with selected members of the protein kinase family.

Figure 20 illustrates the results of expression of CSBP in E. coli.

Figure 21 illustrates the full length nucleic acid sequence of CSBP-1 cDNA.

Figure 22 illustrates the full length nucleic acid sequence of CSBP-2 cDNA.

Detailed Description of the Invention:

In further describing the present invention, the following additional terms will be employed, and are intended to be defined as indicated below.

An "antigen" refers to a molecule containing one or more epitopes that will stimulate a host's immune system to make a humoral and/or cellular antigen-specific response. The term is also used herein interchangeably with "immunogen."

The term "epitope" refers to the site on an antigen or hapten to which a specific antibody molecule binds. The term is also used herein interchangeably with "antigenic determinant" or "antigenic determinant site."

"Fusion protein" is a protein resulting from the expression of at least two operatively-linked heterologous coding sequences. The protein comprising a CSAIDs binding protein or fragment thereof and a second unrelated peptide sequence is an example of a fusion protein.

A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into a single mRNA, which is then translated into a single polypeptide having amino acids derived from both coding sequences. The coding sequences need not be contiguous to one another so long as the expressed sequence is ultimately processed to produce the desired protein.

"Recombinant" polypeptides refer to polypeptides produced by recombinant DNA techniques; i.e., produced from cells transformed by an exogenous DNA construct encoding the desired polypeptide. "Synthetic" polypeptides are those prepared by chemical synthesis.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (bases adenine, guanine, thymine, or cytosine) in a double-stranded helix, both relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA).

A DNA "coding sequence of" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a polypeptide when placed under the control of appropriate regulatory sequences.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at the 3' terminus by a translation start codon (e.g., ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the expression (i.e., the transcription and translation) of a coding sequence in a host cell.

A control sequence "directs the expression" of a coding sequence in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated (covalently linked) into chromosomal DNA making up the genome of the cell. In prokaryotes and yeasts, for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eukaryotic cells, a stably transformed or transfected cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cell containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

Two DNA or polypeptide sequences are "substantially homologous" or "substantially the same" when at least about 85% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. As used herein, substantially homologous also refers to sequences showing identity to the specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., "Current Protocols in Mol. Biol." Vol. I & II, Wiley Interscience. Ausbel, et al. (ed.) (1992). Protein sequences that are substantially the same can be identified by proteolytic digestion, gel electrophoresis and microsequencing.

The term "functionally equivalent" with respect to CSBP intends that the amino acid sequence of the subject protein is one that will display the CSAIDs binding activity disclosed herein.

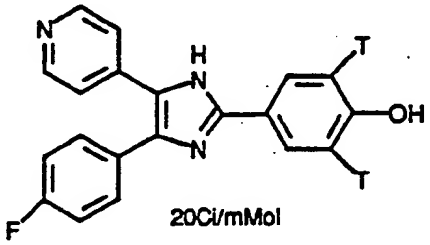
A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a receptor gene, the gene will usually be flanked by DNA that does not flank the gene in the genome of the source animal. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). Allelic variation,

alternative splicing or naturally occurring mutational events do not give rise to a heterologous region of DNA, as used herein.

Development of Molecular Reagents:

Radioligand Synthesis

In order to isolate and purify the CSBP of this invention, it was first necessary to provide several labeled molecular reagents. The phenolic triaryl imidazole, Compound I, was chosen as an alternative radioligand because of its nanomolar potency and the relative ease of synthesis of the radiolabeled compound through catalytic reduction of the corresponding aryl bromide in the presence of tritium gas.

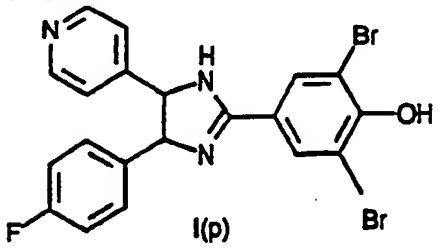


20Ci/mMol

Compound I

IL - 1 IC50=0.05μM

Compound I was prepared according to the following reaction protocol:

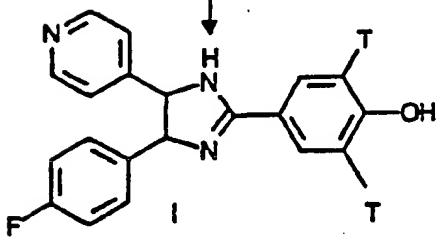


I(p)

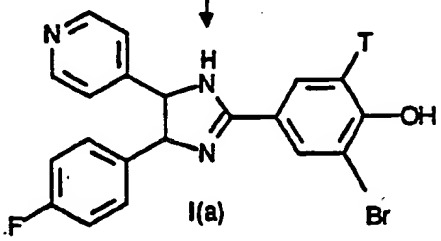
2-(3,5-Dibromo-4-hydroxyphenyl)-4-(4-fluorophenyl)-5-(4-pyridyl)imidazole
MW.489.141
2.9 mg

Tritium gas, 5.3 Ci
Pd/C
DMF, Et_3N

HPLC



I



I(a)

4-(4-Fluorophenyl)-
2-(4-hydroxyphenyl-
3,5-t_2)-5-(4-
pyridyl)imidazole
331.349
48.6 mCi

2-(3-Bromo-4-hydroxy-5-t)
-4-(4-fluorophenyl)-5-(4-
pyridyl)imidazole
M.W. 410.245
10.1 mCi

HPLC

12.2 mCi

4-(4-Fluorophenyl)-
2-(4-hydroxyphenyl-
3,5-t_2)-5-(4-
pyridyl)imidazole
Compound I
9.3 mCi

Preparation of 4-(Fluorophenyl)-2-(4-hydroxyphenyl-3,5-t_2)-5-(4-pyridyl)imidazole, (Compound I).

A 2.9 mg (0.0059 mmol) portion of 2(3,5-Dibromo-4-hydroxyphenyl)-4-(4-fluorophenyl)-5-(4-pyridyl)imidazole, Compound I(p), was dissolved in 0.95 mL of dry DMF and 0.05 mL of triethylamine in a 2.4 mL round bottom flask equipped with a small magnetic stirring bar. A 1.7 mg portion of 5% Pd/C (Engelhard lot 28845) was added, and the flask was attached to the stainless steel tritium manifold. The mixture was degassed through four freeze-pump-thaw cycles, then tritium gas (5.3 Ci, 0.091 mmol) was introduced. The reaction mixture was allowed to warm to room temperature and was stirred vigorously for 20h. The mixture was frozen in liquid nitrogen, the remaining tritium gas (2.4 Ci) was removed, and the flask was removed from the manifold. The reaction mixture was transferred, using 3 x 1 mL of methanol as rinsings, into a 10 mL round bottom flask, and the solvents were removed by static vacuum transfer. A 1.5 mL portion of methanol was added to the residue, then removed by static vacuum transfer. The latter process was repeated. Finally, the residue was suspended in 1.5 mL of ethanol and filtered through a syringe-tip Millipore filter (0.45 micron), along with 3 x ca. 1 mL ethanol rinsings. The total filtrate volume was determined to be 3.9 mL, and the total radioactivity, 94.2 mCi. Solution was determined to be 3.9 mL, and the total radioactivity, 94.2 mCi. HPLC analysis of filtrate (Partisil 5 ODS-3, 4.6 mm I.D. x 25 cm, 1 mL/min of 70:30:01 water/acetonitrile/trifluoroacetic acid, Radiomatic Flo-One Beta radio detector with 3 mL/min of Ecoscint-H cocktail through a 0.75 mL cell) showed the presence of Compound I (R_t = 60 min. ca. 37% of total radioactivity), and a discrete intermediate presumed to be the monobromo derivative Compound Ia (R_t = 11.8 min, ca. 9%).

The filtrate solution was evaporated to near dryness with a stream of nitrogen, and the residue was dissolved in about 1.2 mL of the HPLC mobile phase.

The solution was separated by HPLC as shown below, and the peaks corresponding to Compounds I and Ia and SB collected separately.

HPLC Method	
Column	Altex Ultrasphere 10 mm I.D. x 25 cm
Mobile Phase	70:30:0.1 water/acetonitrile/trifluoroacetic acid
Flow Rate	5 mL/min
UV detection	210nm
Injection Volumes	0.05 - 0.4 m:
Retention Times	7.8 min Compound I 24 min Compound Ia

The pooled Compound I fractions totaled 32 mL in volume and the radioactive concentration was 1.52 mCi/mL (total 48.6 m Ci). The pooled SB Compound Ia [^{3}H] fractions (totaling 10.1 mCi) were evaporated to dryness and the residue was transferred quantitatively into a glass vial using 3.8 mL of absolute ethanol for further analysis.

An 8 mL (12.2 mCi) portion of Compound I was evaporated to dryness *in vacuo* at <35°C, then redissolved in 0.5 mL of mobile phase. The whole volume was injected into the HPLC system described above, and the appropriate peak was collected. Evaporation of the collected eluate *in vacuo* at <35°C and transfer of the yellow residue into a vial with absolute ethanol provided a solution (3.8 mL, 2.44 mCi/mL) of Compound I. The portion of this solution used for NMR analyses was first evaporated to dryness using stream of nitrogen then taken up in CD_3OD.

Analysis of 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl-3,5-t_2)-5-(4-pyridyl)imidazole, Compound I.

Radiochemical Purity by HPLC

Method	
Column	Ultrasphere Octyl, 5µm, 4.6 mm I.D. x 25 cm, Beckman
Mobile Phase	350:150:0.5(v/v/v) water/acetonitrile/trifluoroacetic acid
Flow Rate	1.0 mL/min
Mass detection	UV at 210 nm
Radioactivity detection	Ramona-D radioactivity flow detector
Scintillator	Tru-Count (Tru-Lab Supply Co.)
Flow rate	5.0 mL/min
Cell volume	0.75 mL
Retention time	7.7 min
Result	98.7

Radioactive Concentration by Scintillation Counting

Method	
Scintillator	Ready Safe (Beckman Instruments, Inc.)
Instrument	TM Analytic model 6881
Efficiency	Automated DPM calculation from quench curve
Result	2.44 mCi/mL

Specific Activity by Mass Spectrometry

Method	CI-MS, NH_3 reagent gas	
Result	20.0 Ci/mmol	
	^3H Distribution:	
	Unlabeled	44%
	Single Label	43%
	Double Label	13%

^3H NMR[9]

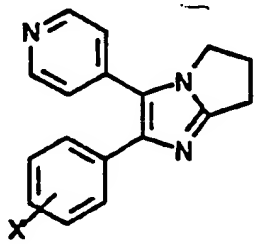
Method	
Instrument	Brunker AM 400
Experiment	Proton decoupled ^3H NMR Proton non-decoupled ^3H NMR Proton non-decoupled ^3H NMR
Peak Referencing	Solvent Peak of methanol ∂ 3.3
Solvent	Methanol-d_4
Result	Tritium is incorporated exclusively on the carbon atoms ortho to aromatic hydroxyl group

Analytical Summary

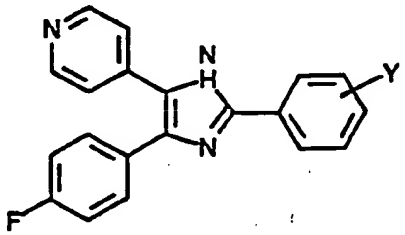
Assay	Result
Radiochemical purity determined by HPLC	98.7%
Radioactivity concentration determined by scintillation counting	2.44 mCi/mL
Specific activity determined by mass spectrometry	20.0 Ci/mmol
^3H NMR	agrees with the proposed structure

Photoaffinity Radiolabeled Ligand

Additionally, a photoaffinity radiolabel was synthesized. Ideally, the radiophotoaffinity reagent should have a submicromolar binding affinity, a convenient site for the attachment of a radiolabel (preferable a gamma emitter) and allow for the positioning of the photoreactive group, (e.g. an azide) proximal to the binding site. The SAR leading to the proposal of Compound IV as the candidate for the photoaffinity reagent is illustrated in Table I below.



Formula II

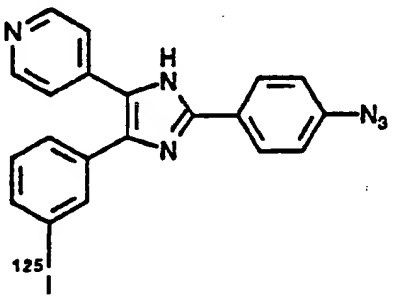


Formula III

Table I

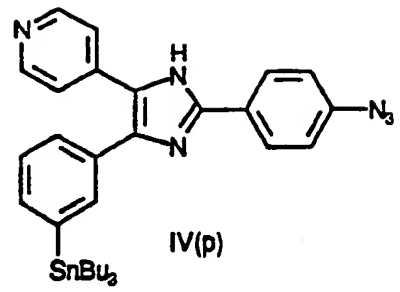
Compound	X	BioAssay IC50, μM	Compound	Y	BioAssay IC50, μM
IIa	4-F	> 0.1	IIIa	H	0.15
IIb	4-H	0.5	IIIb	4-N_3	0.05
IIc	4-Cl	0.05	IIIc	3-I-4-NH_2	0.48
IId	3-Cl	0.04	IIId	4-NH_2	0.28
IIe	2-Cl	0.25			
IIf	4-I	0.58			
IIg	3-I	0.05			

In addition, a specific ELISA assay may also be usefully employed to determine IL-1β and TNFα levels (see: PCT Applications US93/00674 and US93/00675)



Compound IV
CSAIDs binding IC_{50} = 0.72 μM

The synthesis of radioiodinated photoaffinity label, Compound IV, employed a palladium-mediated stannylation of the aryl iodide and subsequent electrophilic radioiodination, according to the following protocol.



IV(p)

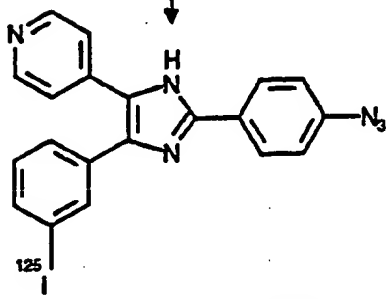
[3-[2-(4-azidophenyl)-5-(4-pyridinyl)-1H-(4-pyridinyl)-imidazol-4-yl]phenyl]phenyl]tributylstannane

M.W.627.40

Compound IV (p)

250 µg

$Na^{125}I$ | Chronic-T, 3% HOAc in EtO



4-[2-(4-azidophenyl)-5-(3-^{125}Iodo-phenyl)-1*H*-imidazol-4-yl]pyridine

Compound IV

3.60 mCi

Process Description

Synthesis and purification of 4-[2-(4-azidophenyl)-5-(3-^{125}Iodo-phenyl)-1*H*-imidazol-4-yl]pyridine.

[3-[2-(4-Azidophenyl)-5-(4-pyridinyl)-1*H* imidazol-4-yl]phenyl]-tributylstannane, Compound IV (p) (250 µg, 0.398 µmol, was dissolved in 100 µL of 3% acetic acid in ethanol. To this solution was added 2.85 µg of chloramine-T hydrate (0.013 µmol) in 11.4 µL of water and 5.19 mCi of sodium [^{125}I]iodine in 45 µL of 0.1 N sodium hydroxide. Another 50 µL of 3% acetic acid in ethanol was added to make

the reaction mixture homogeneous. The reaction was stirred 60 minutes at room temperature (in the dark). The reaction was then blown to dryness under a stream of dry nitrogen and the residue partitioned between chloroform (1 mL) and saturated aqueous sodium bicarbonate (1 mL). The aqueous layer was extracted with chloroform (2 x 1 mL), the organic layers were combined and dried by passing through a pipet filled with granular sodium sulfate. The solvent was removed under stream of dry nitrogen; the residue was found to contain 4.36 mCi of iodine-125 (assayed on the Capintec dose calibrator). The aqueous layers were found to contain 310 µCi of iodine-125. The residue from the organic layer was taken up in 80 µL of HPLC mobile phase and purified on a Baker SiO_2 column, 5µm, 4.6 mm I.D. x 250 mm, eluted at 1.5 mL/min with 90:10:1 (v/v/v) hexane/isopropanol/triethylamine, with UV monitoring at 260 nm. The product fractions were combined and blown to dryness under a stream of dry nitrogen. The product was taken up in 3.0 mL of absolute ethanol. This procedure gave 3.60 mCi of Compound IV at a radiochemical purity of 99.0%, radioactive concentration of 1.20 mCi/mL and a specific activity of 1736 Ci/mmol.

Analysis of 4-[2-azidophenyl)-5-(3-iodo-^{125}I-phenyl)-1*H*-imidazol-4-yl]pyridine, Compound IV.

Radiochemical Purity by HPLC

Method	
Column	Baker, Silica, 5 µm, 120 A, 4.6 mm I.D. x 25 cm.
Mobile Phase	90:10:1 (v/v/v) hexane/isopropanol/triethylamine
Flow Rate	1.3 mL/min
Mass detection	UV at 260 nm
Radioactivity detection	
Detector	β-RAM radioactivity flow detector
Scintillator	Tru-Count (Tru-Lab Supply Co.)
Flow rate	5.0 mL/min
Cell size	0.8 mL
Retention time	17.0 min
Result	99.0%

Mass Concentration by HPLC

Method	Baker, Silica, 5 µm, 120 A, 4.6 mm I.D. x 25 cm.
Column	
Mobile Phase	90:10:1 (v/v/v) hexane/isopropanol/triethylamine
Flow Rate	1.5 mL/min
Mass detection	UV at 260 nm
Retention time	11.2 min
Result	99.0%

Radioactive Concentration by Scintillation Counting - external standard method

Method	
Solvent	Ready Safe (Beckman)
Instrument	TM Analytic model 6881
Efficiency	Automated DPM calculation from quench curve
Result	1.2 mCi/mL

Specific Activity Derived from Mass and Radioactive Concentrations

Method	derived from mass and radioactive concentrations
Result	1736 Ci/mmol

Analytical Summary

Assay	Result
Radiochemical purity by HPLC	99.0%
Massive concentration by HPLC	0.32 µg/mL
Radioactive concentration	1.2 mCi/mL
Specific activity derived from mass and radioactive concentrations	1736 Ci/mmol

The photoaffinity label has an IC_{50} of 0.5-0.8 µM in a competitive binding assay and IC_{50} of 3µM in a CSAIDs bioassay.

CSAIDs Bioassay

The biological assay employed to evaluate CSAIDs activity was the IL-1 dependent EL-4/IL2 induction assay (Simon, P. L. et al., J. Immuno. Meth. 84: 85-94 (1985)). Briefly, Human monocytes were plated in 24-well plates in LPS-free RPMI 1640 media containing 1% human AB serum at a concentration of 10^6

per millilitre per well and allowed to adhere for 1 h at 37°C; non-adherent cells were removed by gentle washing. Test compounds or media were added to the cells 0 or 1 h before the addition of bacterial lipopolysaccharide (E. *coli* 001:B4; Difco, Detroit) at 10 ng/ml. The cultures were then incubated at various intervals as indicated at 37°C in a humidified 5% CO_2 atmosphere. At the end of the incubation period, culture supernatants were collected. The residual adherent monocytes were lysed in a buffer containing 0.15M octyl-glucopyranoside, 25 mM Hepes, and 0.5mM phenylmethylsulfonylfluoride in saline. Both supernatants and cell lysates were clarified by centrifugation and assayed for IL-1 activity.

IL-1 activity was measured by its ability to stimulate the secretion of IL-2 by EL-4 (ATCC TIB181) cells in the presence of A23187 ionophore. Serial dilutions of the samples were incubated with 10^5 EL-4 cells in the presence of 2×10^{-7} M calcium ionophore A23187. After overnight incubation, 0.1 ml of a cell-free supernatant from each culture was taken and incubated with 10^4 IL-2-dependent CTLL-20 (ATCC-TIB214) cells. Following an additional 20 hours of incubation, the cultures were pulsed with 1 μCi of tritiated thymidine for 4 h. The cells were then harvested onto glass-fibre filters and the radioactivity determined by liquid scintillation counting. All determinations of IL-1 activity were made in comparison to a standard.

CSAIDs Binding Assay

The next phase of the isolation and purification of CSBP required the development and validation of a cell-based CSAIDs binding assay. As mentioned above the early CSAID studies were conducted in human monocytes. A more convenient cell source, the human monocytic leukemia cell line, THP.1, (ATCC TIB 202) was selected and was shown to be an adequate surrogate cell source for mechanistic studies by virtue of its response to stimuli to produce IL-1 and TNF as well as a sensitivity towards CSAIDs comparable to human monocytes (Figure 1).

Radiolabeled Compound I was taken up by intact THP.1 cells in a time-dependent manner (Figure 2). The uptake of the radiolabel was rapid and reached a maximum level at 3-5 minutes at 37°C. In addition, the uptake of radiolabel was saturable and specific.

Upon subcellular fractionation of radiolabeled ligand loaded THP.1 cells, the predominant subcellular site of accumulation of the radioactivity was found to be the cytosol. (Figure 3).

A specific and reproducible CSAID binding assay was developed using soluble cystosolic fraction from THP.1 cells and radiolabeled Compound I. In brief, THP.1 cytosol was routinely prepared from cell lysate obtained by nitrogen cavitation followed by a 10 K x g low speed and a 100 K x g high speed centrifugation, the supernatant of which was designated as the cytosolic fraction. THP.1 cytosol was incubated with appropriately diluted radioligand at room temperature for a pre-determined time to allow the binding to achieve equilibrium. The sample was added to a G-10 column and eluted with 20 mm TRN, 50μMβ - mercaptoethanol, NaN_3. The fraction encompassing the void volume was collected and the radioactivity was assessed by liquid scintillation counting. This was determined to reflect bound radioligand since the radioactive signal was abrogated by the presence of excess cold ligand in the incubation mixture or when there was no cytosolic fraction present.

More specifically, the CSAID Binding Assay is performed as follows:

Materials:

Incubation buffer: 20 mM Tris, 1 mM $MgCl_2$, 20 μM Hepes, 0.02% NaN_3, store at 4°C. Elution buffer: 20 mM Tris, 50 μM 2-mercaptoethanol, NaN_3, store at 4°C.

G-10 Sephadex: add 100 g Sephadex G-10 (Pharmacia, Uppsala, Sweden) to 400 mL dd H_2O and allow to swell at room temperature for 2 hours. Decant fines and wash 3 times. Add NaN_3 and QS with dd H_2O to 500 mLs and store at 4°C.

Assemble Columns: Straw column, filter frit and tip (Konotes, SP 420160-000, 420162-002). Lowsorb tubes (Nunc) used in binding reaction. THP.1 cytosol spun at 15000 rpm for 5 min to clarify. THP.1 cytosol prepared by hypnotic treatment of cells and lysis by decompression in nitrogen. Nuclei and membrane fragments removed by differential centrifugation (10,000 g for 1 hour and 100,000 g for 1 hour).

Compounds: Non-radioactive Compound I with corresponding EtOH control (dilutions made in incubation buffer) and ^3H-Compound I (dilutions in incubation buffer).

Method:

A. Column Preparation

1. Begin 30 min before anticipated elution of reaction mixture.
2. Add 3 mL of G-10 slurry to column for bed vol of 1.5 ml.
3. Rinse with 7 mL elution buffer (fill to top of column)
4. Cut columns down to size.

B. Sample Incubation

1. 15 min incubation at 4°C.
2. Binding reaction mixture; 100 µL cytosol, 10 uL cold Compound I or EtOH control, 10 µL ^{3}H-Compound I (molar concentration depends on nature of study).
3. "Free" control = 100 µL incubation buffer in lieu of cytosol preparation.

C. Sample Elution

1. Elute at 4°C.
2. Add total reaction volume to G-10 column.
3. Add 400 µL elution buffer to column and discard eluate.
4. Add 500 µL elution buffer to column, collecting eluted volume in 20 ml scintillation vial.
5. Add 15 mL Ready Safe scintillation fluid.
6. Vortex and count in liquid scintillation counter for 5 minutes. Include a "total input counts control" (10 µL of labeled ligand).

D. Data Analysis

1. Plot DPMS as ouptut in graphic form and analyze by regression analysis and "Lundon ligand binding" software for the determination of IC 50 and Kd/Ki respectively.
2. Rank order the IC50s of the tested compounds in the CSAID bioassay and compare to that generated by the CSAID binding assay and establish a correlation curve.

The binding assay was further validated by the following criteria:

- THP.1 cytosol demonstrated saturable and specific binding of radiolabeled Compound I (Figure 4).
- A substantial number of pyridinyl imidazole CSAIDs were tested in the radiolabel competitive binding assay. The rank order potency and the IC50s of the compounds was highly correlative to that determined by the human monocyte bioassay (Figure 5). Furthermore, the competitive binding activity was regioselective (Figure 6). These results underline the particular usefulness of the binding assay to the cytokine suppressive effects of these compounds and is considered particularly advantageous for SAR development and providing the means to help eludicate the molecular target.
- Binding is highly specific for the pyridinyl imidazole CSAIDs. A series of non-structurally related compounds of varied pharmacological activities were tested in the competitive binding assay. They include the specific cyclooxygenase inhibitors, 5-lipoxygenase inhibitors, dual CO/LO inhibitors, PDE IV inhibitors, immunosuppressive macrolides, steroids, and others (Table II). None of the compounds tested at 100 μM demonstrated competitive binding.

A list of non-pyridinyl imidazole CSAIDs, related anti-inflammatory or immunosuppressive compounds tested in the competitive CSAID binding assay is provided in Table II. Unless otherwise indicated, no competitive binding was observed up to 100 μM.

TABLE II

Cyclooxygenase Inhibitors • Indomethacin • Naproxen	Steroid • Dexamethasone
Selective 5-Lipoxygenase Inhibitors • Hydroxyurea class • Aminophenol class	Novel Anti-Inflammatories • DX270 • Tenidap (IC50 = 139 μM) • Romazarit

5-Lipoxygenase Translocation Inhibitor • MK886	Peroxisome Proliferators • Wyeth 14643 Clofibrate
Dual Inhibitors • Phenidone • NDGA (IC50 = 154 μM)	AH Receptor Agonists • 3-Methylcholanthrene • βNaphthoflavone
Immunosuppressives • FK506 • Azaspirane • Rapamycin & Analogs	Miscellaneous • Tibenelast • Tetrandrine
PDE_{IV} Inhibitor • Rolipram	

Having established a cell source and a binding assay, further characterization of CSBP established that the CSAID binding is saturable, specific and reversible (Figure 7), follows a rapid on and off rate, the binding activity is sensitive to protease and heat treatment (Figure 8) and is protein concentration dependent (data not shown).

The CSAID binding activity in human monocytes is indistinguishable from that determined for THP.1 by the criteria established for the binding activity listed above.

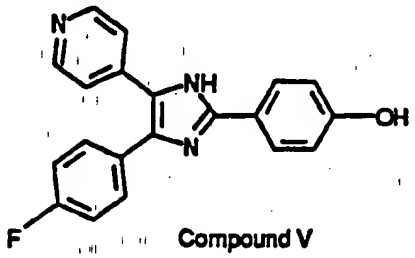
The binding is pH dependent with an optimal pH range from 5 to 8 and is independent of divalent cations and is sensitive to high salt concentration which is reversible.

Purification of CSBP

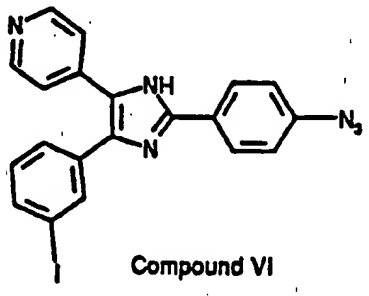
The purification of the CSBP from THP.1 cells was accomplished as follows:

Materials

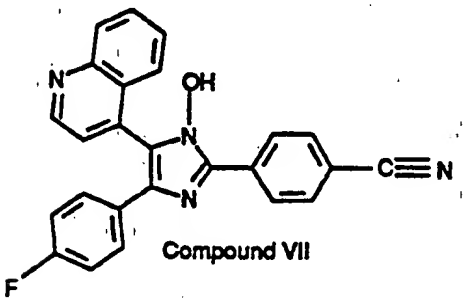
The following compounds were synthesized by the methods outlined in PCT application, US93/00674 and US93/00675 both filed January 13, 1993.



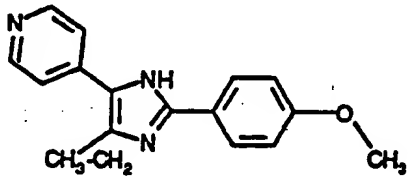
Compound V



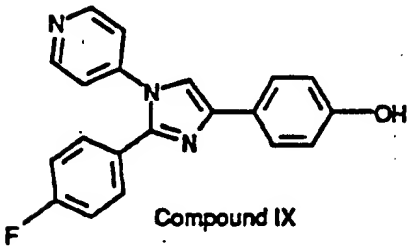
Compound VI



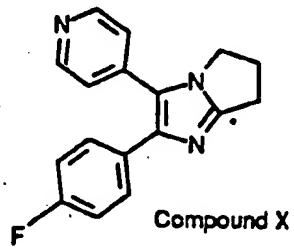
Compound VII



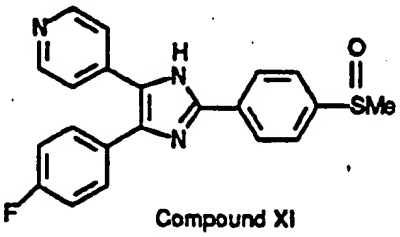
Compound VIII



Compound IX



Compound X



Compound XI

The radiolabeled compounds II and IV were prepared as described above. Polyclonal and monoclonal antibodies against actin (rabbit (cat #65-096) and mouse (cat. #69-100), respectively) were purchased from ICN Biomedicals. The peptide NH_2-Ile-Thr-Ala-Ala-Gln-Ala-Leu-Ala-His-Ala-Tyr-Phe-Ala-Gln-Tyr-Cys-COOH (Seq. I.D. No. 1) was synthesized by standard solid phase FMOC chemistry (see for example: Fields, G.B., et al. Int'l. Peptide Protein Res. 35: 161-214 (1990), purified and coupled to maleimide activated keyhole limpet hemocyanin (KLH) (Pierce Chemical Co. Cat # 77105A) by conventional methods, and used to inoculate rabbits. All other chemicals were of reagent grade and unless otherwise specified, were not purchased from a particular vendor.

Growth of THP.1 Cells

THP.1 cells were grown and processed as follows:

THP. 1 cells are grown in RPMI - 1640 medium with 25 mM Hepes, 10% FBS (8% in reactors), 10mM glutamine, and .05% pluronic F-68. The cells were passed on a 3/4 days cycle with an average cell count of 2×10^6 (seeding density between 2×10^5 and 3×10^5). A high density cell recycle in shake flasks was used to scale-up the cells to the large reactors. In this process, the total volume of the shake flask was spun down and resuspended with the same volume of fresh medium. Therefore, seeding density increased with each passage, giving a higher density of cells per volume. The densities ranged from 6×10^6 to 12×10^6.

From the shake flasks, two scale-up procedures were used to obtain the required volumes. Initially, two 80L artisan reactors (60L working volume) were used. Every five days, 50L was taken out of both reactors and harvested. The cells were then fed with an additional 50L until the total required volume was reached. Alternatively, cells were grown in a 30L artisan and used to seed the 250L Abec reactor (totaling working volume was 150L). 120L was harvested every five days and the 30L left was refed. The seeding density was between 3×10^5 and 5×10^5. The pH for both types of reactors was controlled between 7.0 and 7.2. CO_2 was used as the controlling acid and sodium bicarbonate as the buffer. The D.O. was set at 30 percent for the Artisans reactors and 20 percent for the Abec reactor.

Preparation of THP.1 Cytosol

Cells were lysed by nitrogen cavitation in 20 mM TrisHCl pH 7.4, 1mM $MgCl_2$, 1mM PMSF, 1μM pepstatin A and 1μM leupeptin. Insoluble material was pelleted at 10,000 x *g* for 10 min and the supernatant further clarified by a 100,000

x *g* centrifugation for 1 h at 4°C. The supernatant from the final centrifugation was collected and is hereafter referred to as the THP.1 cytosol.

Measurement of CSAID Binding Activity

The same (typically 200 µg protein) was incubated with appropriately diluted ^3H - Compound I (50 nM) at room temperature for 60 min to allow the binding to achieve equilibrium. Free ligand was separated from bound ligand on a 1.5ml Sephadex G-10 column in 20 mM TrisHCl pH 7.4. The fraction encompassing the void volume was collected and the radioactivity was assessed by liquid scintillation counting. Protein concentrations were determined by the bicinchoninic acid assay (Pierce).

Superose 12 Chromatography

Approximately 100 to 250 ml of THP.1 cytosol was applied at 14.5 cm h^{-1} to a 5 L Superose 12 column (Pharmacia; 11.5 x 50 cm) equilibrated in 10 mM $NaPO_4$ pH 7.0 and 150 mM NaCl at 4°C. Fractions were collected (50ml) and assayed for CSAID binding activity; a single peak of activity corresponding to an elution volume for a protein of M_r ~ 50,000 was pooled (200 to 500 ml).

Hydroxylapatite Chromatography

The material from the Superose 12 column were applied at 30 cm h^{-1} to a 160 ml Hydroxylapatite HA column (Cal. Biochem; 5.0 x 8.0 cm) equilibrated in 10 mM $NaPO_4$ pH 7.0 at room temperature. The column was eluted with a 10 to 200 mM $NaPO_4$ gradient over 2.5 column volumes. Fractions (30 ml) were collected and assayed for CSAIDs binding activity. A protein peak containing approximately 60% of the CSAID binding activity applied to the column was pooled (50 to 250 ml).

Radiophotoaffinity Labeling of CSBP

The following protocol was used for about 30 ml of sample but can be adapted for larger or smaller volumes. The hydroxylapatite pool was concentrated to about 30 ml using an Amicon stir cell (YM30 membrane, 70 psi N_2). Insoluble material in the concentrate was removed by centrifugation (10,000 x *g* for 30 min in SS34 rotor at 4°C). The supernatant (450 mg protein) was used in the labeling reactions, which were performed in 6-well microtiter plates (Nunc). Six reactions were carried out using the following reagents and protocol. Approx. 60 mg. of

protein (4 ml) was added to 0.25 ml buffer (10 mM $NaPO_4$ pH 7.0, 150 mM NaCl) and 0.25 ml 50 nM radioactive (i.e. "hot") ^{125}I Compound IV (final concentration of 2.5 nM, 250 μCi) in dim light and allowed to stand on ice for 10 to 15 min. The microtiter plate was exposed to > 300 nm light at a distance of 5 to 10 cm for 2 min while on ice. The reactions were chased with Compound IV (Compound VI being the "cold" (i.e. non-radioactive) form of Compound IV) as follows. A 1 mM stock of Compound VI was prepared by adding 0.3 ml 10mM Compound VI to 2.7 ml 50% ethanol in 10 mM $NaPO_4$ pH 7.0 and 150 mM NaCl. Compound VI (0.5 ml 1 mM) was added to each labeling reaction in dim light and allowed to stand for 10 to 15 min on ice. The reactions were exposed to light as for the radioactive labeling. Unreacted Compounds IV and VI can be removed from labeled protein by the preparative isoelectric focusing or electrophoresis steps; or for samples of smaller volume, removed by gel filtration chromatography on Sephadex G-25 (1.6 x 12 cm) in 20 mM $NaPO_4$ pH 7.4 and 150 mM NaCl.

Analytical Electrophoresis, Autoradiography and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions essentially as described by Smith B.J., Meth. in Mol. Biol., Vol I, pp. 44-57 (1984). Samples were run on 0.75 mm thickness 16 cm (4% stacking, 10 or 12% separating) or 10 cm (12% pre-cast, Jule) stab gels using the Hoefer SE 600 or Mighty Small electrophoresis systems, respectively. Protein was stained by either coomassie blue R350 (Pharmacia) or silver (Silver Stain Plus, BioRad). Molecular weight protein standards were purchased from Amersham or BioRad. For blotting, proteins were transferred to a polyvinylidene difluoride membrane (Millipore) in 192 mM glycine/25 mM Tri pH 8.3 and 20% (v/v) methanol using a Genie electrophoretic blotter (Idea Scientific) at 15 V. Protein labeled with ^{125}I was visualized by autoradiography using Hyperfilm-MP (Amersham) after overnight exposures at -70°C. The membrane was blocked with 5% gelatin in 20 mM TrisHCl pH 7.5 and 500 mM NaCl before incubation with the appropriate antiserum diluted 1,000 to 5,000-fold in buffer. The antibody complexes were detected with anti-mouse or anti-rabbit immunoglobulin G (Amersham) coupled to horse radish peroxidase and visualized by luminol phosphorescence on Hyperfilm-ECL (Amersham).

Preparative Isoelectric Focusing

Preparative isoelectric focusing was performed using a Rainin RF3 recycling free flow focusing protein fractionator at 4°C overnight, concentrated to about 3 ml with an Amicon stir cell (YM30 membrane, 70 psi N_2), and brought to 10% glycerol and 1% ampholyte (Pharmacia Ampholine or Pharmalyte pH 4 to 6) for a final volume of about 10 ml. Before the sample was applied to the RF_3, a 1% ampholyte/10% glycerol solution was pre-focused for 1 to 1.5 h (until the voltage, current, power and temperature were at baseline). The sample was injected into bubble port 14 using a needle and syringe. The system was allowed to equilibrate as for the pre-focusing before collecting 3 ml fractions. Labeled CSBP was identified by monitoring the radioactivity, and the appropriate fractions pooled.

Preparative SDS-Page

Preparative SDS-PAGE was performed using the BioRad Model 491 Preparative cell. The pooled fractions from the preparative isoelectric focusing were concentrated to 2 to 3 ml with an Amicon stir cell (YM30 membrane, 70 psi N_2). Approximately 2 to 2.5 ml of the concentrate was brought to about 3 ml in 100 mM Tris pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol, 10% glycerol and 0.01% bromophenyl blue before incubating at 100°C for 3 to 5 min. The sample was applied to the gel (2 cm 4% stacking gel, 6 cm 11% separating gel) and run at 40 mA in 192 mM glycine/25 mM Tris pH 8.3 and 0.1% SDS at room temperature. Fractions (2.5ml) were collected and assayed for radioactivity in order to identify where labeled CSBP eluted from the gel.

Results

Partial Purification of CSBP

A typical partial purification of CSBP from THP.1 cytosol is summarized in Table III. As indicated, the recovery of activity is 20% and the level of purification is 3-fold. This was characteristic of CSBP recovery and purification during evaluation of a number of chromatography resins (anion and cation exchange, hydrophobic interaction with $(NH_4)_2SO_4$, blue sepharose, heparin sepharose, etc.); the purification scheme as listed in the Table III gave the best recovery and most reproducible results. Since attempts to purify CSBP further while following CSAID binding activity resulted in poor recovery of activity, this was as far as the purification was taken before photoaffinity labeling.

Table III

Purification of CSBP from THP.1 cytosol

Sample	Activity, dpm[a]	Protein, mg	Specific Activity, dpm mg^{-1}
THP.1 cytosol[b]	5.0×10^8	6800	7.4×10^4
Superose 12	1.6×10^8	1200	1.3×10^5
Hydroxylapatite	9.6×10^7	500	1.9×10^5

[a]activity is expressed as the ^3H radioactivity (disintegration per minute, dpm) collected in the CSAID binding assayed as described above and corrected for the total sample.

[b]THP.1 cytosol was prepared from starting material equivalent to approximately 10^{11} cells.

Photoaffinity Labeling of CSBP

CSBP was covalently labeled with the ^{125}I, aryl azide CSAID derivative Compound IV. The reaction was very specific as illustrated in Figure 9, which shows that a single protein of M_r 43,000 was labeled (the lanes labeled "None"). During the partial purification described above the CSAID binding activity eluted as a single peak from the Superose 12 gel filtration chromatography with a molecular weight corresponding to a protein of M_r 45,000 to 50,000. Collectively, these two analyses indicate the CSBP is a single-chain, or "monomeric" protein of M_r 43,000.

Figure 9 also illustrates the specificity of the labeling. In the middle lanes of the gel, protein was preincubated with a non-radioactive CSAID (10 μM) before the photoaffinity labeling with ^{125}I Compound IV (2.5 nM). The extent to which each CSAID competed with the photoaffinity label correlates well with its potency in a cellular assay. That is, the more potent the compound is in its ability to suppress IL-1 production in human monocytes, the more effectively it prevented photaffinity labeling of the CSBP. Thus, CSBP is the protein labeled with Compound IV.

Purification of Labeled CSBP

In order to identify CSBP by its amino acid sequence, the labeled protein was further purified from the partially-purified CSBP used for photaffinity labeling. The strategy to accomplish this was preparative isoelectric focusing, preparative SDS-PAGE and reversed-phase HPLC. The results of the preparative isoelectric

focusing are shown in Figure 10. The isoelectric point of the labeled protein corresponded to a pH of about 4.5. Western analysis indicated that some, but not all, of the actin was removed by this procedure. In addition, almost 70% of the protein applied eluted with the labeled protein (50% recovery of radioactivity). This was also demonstrated by SDS-PAGE and silver staining analysis (data not shown). Thus, for this application preparative isoelectric focusing did not provide a substantial purification of the desired protein.

The most substantial purification of labeled CSBP was obtained by preparative SDS-PAGE. The material pooled from preparative isoelectric focusing was applied to a gel using the BioRad Model 491 Preparative Cell. As illustrated in Figure 11, the radioactive fraction corresponding to a protein of about 43 kDa (fraction 56) has at least 90% of the non-radioactive protein removed by this procedure. In addition, unincorporated label is also removed.

Characterization of CSBP

After preparative SDS-PAGE, labeled CSBP was applied to reversed-phase HPLC, where a protein peak coeluting with the radioactivity was collected. Comparison of the protein concentration (determined by amino acid analysis) to the specific radioactivity of the sample demonstrated that only 10% of this protein was labeled (assuming a protein M_r of 43,000). N-terminal sequence analysis identified actin sequences corresponding to 30 to 40 amino acids downstream from the expected amino terminus. Internal sequence analysis following fragmentation with trypsin or CNBr generated approximately 90% actin sequences, but about 10% of the pepides gave unique sequences. One of the sequences from the tryptic digest had strong (85%) homology, but was not identical, to a C-terminal sequence found in a family of Ser/Thr protein kinases known as the mitogen-activated (MAP) kinases (Figure 12; See also: Ray, L.B. & Sturgill, T.W., Proc. Nat'l. Acad. Sci. (USA), 85:3753-3757 (1988)).

A peptide based on the sequence with homology to the MAP kinases was synthesized and used to inoculate rabbits for the production of antisera. Western analysis and autoradiography of labeled THP.1 cytosol 2-D gels demonstrated that 1) antibodies against actin or MAP kinases did recognize proteins on the blot, but not the radiolabeled protein; 2) the antibody prepared from the tryptic peptide recognized the radiolabeled protein. Thus, CSBP appears to have homology to, but is distinct from, the MAP kinases. Given the role of kinases in regulating translation (Pelech and Sanghera, Science 257:1355-66 (1992)) and the effect of

CSAIDs n IL-1 and TNF translation, a kinase is not inconsistent as the molecular target for CSAIDs.

Isolation and Characterization of the CSBP gene:

This invention provides an isolated nucleic acid molecule encoding the human CSBP. Two amino-terminal peptide sequences were obtained from the protein fraction comigrating with the radioactive photoaffinity probe. One of these was derived from a trypsin digest of the radioactive protein fraction but was not itself radioactive, and had the sequence:
ILE THR ALA ALA GLN ALA LEU ALA HIS ALA TYR PHE ALA GLN TYR (Seq. I.D. No. 1)

The second was obtained from an 8KDa cyanogen bromide fragment associated with radioactivity and had the sequence:

XXX (GLN) LEU LEU ASN ASN ILE (VAL/PHE) LYS (PHE) GLN LYS LEU THR (Seq. I.D. No. 2)

where ( ) represents an uncertain assignment and / represents an uncertainty between two amino acids. XXX is an unknown amino acid. A search of Genbank indicated that peptide sequence I.D. No. 1 was homologous to the MAP kinase family of protein kinases, whereas peptide sequence I.D. No. 2 was unique. Based on these two sequences, two degenerate oligonucleotide DNA probes were synthesized using the genetic code to reverse translate the protein sequences, and tables of mammalian cell codon preferences (Grantham, R. et al., Nucl. Acid Res. 9: (1981)).

1. GCYCAYGCTAYTTYGCYCARTA (Seq. I.D. No. 3) and
2. AAYAAYATYKTBAARTTYCAAA (Seq. I.D. No. 4)

where Y = C or T
R = A or G
K = G or T
B = G, C or T

Hence the two mixed oligonucleotides consist of 128 and 384 unique sequences respectively. A cDNA library made from human monocytes treated with GM-CSF (Livi, G.P. et al., Mol. Cell Biol. 10: 2678-86 (1990) in the commercial vector

λZAP (Stratagene) which was screened at low stringency by hybridization to a 50:50 combination of the two synthetic oligonucleotide mixtures labeled with γ-32 P ATP. Labeling of the oligonuleotides followed published methods (Current Protocols in Molecular Biology), typically labeling 3 μg of mixed oligonucleotide with 250 μCi γ-^{32} P ATP and using all of this in a 250 μl hybridization volume. The manufacturer's recommended conditions for plating and lifting phage were followed (see Stratagene λZAP protocol, Stratagene, La Jolla, Ca.) using the BB4 host strain. One additional step was to prewash the filterlifts at 65°C in 2xSSPE/0.1%SDS twice for 30 min. prior to prehybridization to remove bacterial debris.

Subsequently, prehybridization and hybridization with the labeled oligonucleotide probes were performed at 37°C for 24-72h in 6xSSPE, 5xDenhardt's solution, 0.1% SDS and 100 μg/ml phenol/chloroform extracted yeast tRNA. (20xSSPE is 3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA pH7.4.50 x Denhardt's solution is 10 g polyvinylpyrrolidone (MW 40,000), 10g Bovine serum albumin and 10 g Ficoll 400 per liter H_2O.

After hybridization the filters were washed twice under each of the following conditions.

1. 6xSSPE, 0.1% SDS, room temp, 10-15 min.
2. 6xSSPE, 0.1% SDS, 37°C, 10-15 min.
3. 3M tetramethylammonium chloride solution (500g Me_4NCl, 1.38 liter H_2O, 73 ml 1M tris pH 8.0, 5.8ml 0.5M EDTA, 7.3ml; 20% SDS filtered through 0.45 μM filter), 37°C, 30 min (see: Proc. Nat'l. Acad. Sci. USA 82: 1585-1588 (1985) for a description of this technique).

Filters were exposed to Kodak film for 3-5 days in the presence of intensifying screens, and overlapping positives in duplicate filters picked and cycled through the same procedure until pure plaques obtained.

Phage was excised with M13 helper phage R408 in the recA$^-$ E. coli host XL-1 blue according to manufacturers procedures (Stratagene).

After two subsequent rounds of replating and hybridization of positively hybridizing plaques using just the oligonuclcotide mixture #1, a single homogeneous phage was obtained which hybridized in a Southern blot with the oligonucleotide #1 (Seq. I.D. No. 3) but not with oligonucleotide #2 (Seq. I.D. No. 4). Sequencing of the DNA insert of this phage revealed an open reading frame at

one end which encoded part of the No. 2 unique peptide sequence. I.D. No. 2 above. The amino sequence so encoded was:

Asn Ile Val Lys Cys Gln Lys Leu Thr. (Seq. I.D. No. 5).

The rest of the open reading frame (Figure (13) Seq. I.D. No. 6 and 7 was homologous to several protein kinases including the cdc2 and the MAP kinase families. Based on this homology, it is predicted to be missing approximately 130 amino acids from the amino terminus which is obtained via a second round of library screening with the amino terminal region of the obtained cDNA clone.

The other end of the cDNA contains the poly A sequence corresponding to the 3' terminus of the mRNA from which it was obtained (Figure 14, Seq. I.D. No. 8).

Accordingly, based on initial cDNA (Figure 13), oligonucleotides (5'-CCTCGGAGAATTTGGTAGATAAGG-3' (Seq. I.D. No. 9) and 5'-AACATTGTGAAATGTCAGAAGCTTACAGATGACCAT-3' (Seq. I.D. No. 10)) were designed from the 5' end of the sense strand, and used to screen for cDNAs encoding the amino terminus of CSBP. The oligonucleotides were labelled at their 5' ends with polynucleotide kinase and γ-^{32}P-ATP. 10^6 plaques from a GM-CSF stimulated human monocyte library constructed in λZAP were screened on duplicate nitrocellulose filters which had been prewashed prior to hybridization in 2xSSPE, 0.1% SDS at 50°C. After blocking for 48h with 50% formamide, 6 x SSPE, 5 x Denhardt's and 100 µg/ml sheared, denatured salmon sperm DNA, filters were hybridized in the same buffer with the above labelled oligonucleotides for 24 hours at 42°C. The filters were then washed twice with 2 x SSPE, 0.1% SDS at room temperature, followed by two washes in 1 x SSPE, 0.1% SDS at 42°C and two washes in 0.5 x SSPE, 0.1% SDS at 42°C before detection of hybridizing plaques by autoradiography. Positive plaques which appeared on duplicate filters were picked and replated and the procedure repeated twice until unique plaques could be isolated and phagemid DNA released according to manufacturer's protocol (Stratagene Cloning Systems, LaJolla, CA). The cDNAs were sequenced on an Applied Biosystems automated DNA sequencer (ABI 373A) using universal and specific oligonucleotide primers and Taq polymerase cycle sequencing, and the sequences merged and examined using Lasergene software on a Macintosh IIci. Both strands were completely sequenced at least once in each cDNA clone.

Description of cDNAs.

A summary of the cDNAs isolated is illustrated schematically in Figure 15. There are four different cDNAs which have been completely sequenced and are identical in regions of overlap, with one excepti n to be described below. BP01/02 is the cDNA first isolated above, the partial sequence of which is given in Figures 13 and 14. The longest cDNA is 3.8 kb long (N5) Seq. I.D. No. 11 and contains 370 nucleotides of 5' untranslated sequence, a 1.1kb coding region and 2.4kb of 3' untranslated sequence. The extreme 3' end is terminated by a poly A stretch characteristic of mRNA, and is preceded by the expected consensus sequence for polyadenylation. The N7 cDNA has a 3' untranslated region of only 1.4kb terminating in a site and poly A run suggesting an alternative polyadenylation site. On a Northern blot a probe derived from the coding region hybridizes to an ca. 4.2kb mRNA suggesting that the longest cDNA isolated is close to full length.

The coding translates into a protein of 360 amino acids with calculated molecular weight of 41.5 kDA, matching the size of the protein identified by photoaffinity crosslinking with ^{125}I-labelled Compound IV (Figure 16). The predicted isoelectric point (ca. 5.6) is also close to that observed (Ca. 5.0). Examination of the sequence indicates that it contains both the tryptic peptide sequence ITAAQ... and the cyanogen bromide sequence LNNIVK...(boxed) obtained by sequencing of the CSAIDs binding protein in THP.1 cells. These sequences are preceded by the appropriate cleavage sites (arrows). The predicted size of the cynaogen bromide fragment (8kDa) matches the size of the fragment which remains associated with the ^{125}I-labelled radiophotoaffinity label [Compound IV] after cyanogen bromide treatment of the CSAIDs binding protein.

The N13 cDNA (Figure 15) Seq. I.D. No. 12 is identical to the other three cDNAs with the exception of a 75 nucleotide region starting at position 1054 of the N5 cDNA. This difference results in a protein of identical size with amino acids 230-255 altered. (Figure 17). The two different sequences are 43% identical at the nucleotide level, and 44% identical at the amino acid level. Without wishing to be bound by any particular theory, it is likely that the two variants result from alternative internal exon splicing, although allelic variation cannot be excluded. For ease of description, two proteins are referred to herein as CSBP1 (corresponding to the N5 cDNA) and CSBP2 (corresponding to the N13 cDNA).

Comparison of the CSBP sequence to proteins in the GenBank/EMBL or Swissprot databases indicated close homology to a family of proteins known as MAP (Mitogen Activated Protein) or erk (extracellular regulated) kinases (Boulton, et al., "Erks; A Family of Protein Serine-Threonine Kinases that are Activated and Tyrosine Phosphorylated in Response to Insulin and NGF", Cell, 65: 663-675 (1993). This family of protein kinases is conserved from yeast to man as indicated in the phylogenetic tree in Figure 18 with the closest published homologue being the yeast HOG1 gene (Brewster et al., Science 259: 1760-63 (1993). An alignment of the CSBPs with selected members of this family (Figure 19) shows a conservation of all 11 protein kinase motifs (I through XI), including residues identical in all protein kinases (bold) (Hanks et al., Science, 241: 42-52 (1988). Two boxed motifs in regions VI and VIII indicate that the kinases phosphorylate serines and threonines (Hanks et al., 1988). Hence the CSBPs are protein kinases.

A threonine and tyrosine in a TxY sequence (asterisks) proximal to domain VIII are known to be regulatory phosphorylation sites for Erk 1 and Erk 2 (Payne, et al., EMBO. J., 10: 885-892, 1991). These two residues are phosphorylated by MEK (MAPK or ERK Kinase) in response to various extracellular signals, resulting in an activation of the serine/threonine kinase activity of the MAP kinases (Kosako, et al., EMBO. J., 12: 787-794 (1993). The conservation of these amino acids in the CSBPs suggests that they are also regulated by a MEK in response to extracellular stimuli such as LPS. These findings suggest that the CSBPs lie within a cascade of protein phosphorylation events which communicate cell surface stimuli to events such as translational regulation, within the cell. Much of the behavior of the CSBP in suitably stimulated cells can be predicted based on analogy with the known properties and behavior of the MAP kinases (Marshall, et al., Curr. Opin. Genetics & Develop., 4: 82-89 (1994).

A multiple tissue Northern blot with a coding region cDNA probe suggests expression of CSBP mRNA in most tissues. A Southern blot at high stringency (0.1% SSPE, 0.1% SDS) suggested a single gene; however lower stringency washes may reveal closely related kinases. Gene mapping experiments using a panel of human/mouse hybrid cell lines available commercially indicated that the gene for CSBP resides on human chromosome 6.

Expression in E. coli

To confirm that the proteins encoded by the isolated cDNAs can bind to CSAIDs, the cDNAs were expressed in E. coli and yeast. In E. coli the CSBPs were expressed as fusion proteins with β-galactosidase and/or an enterokinase cleavable FLAG epitope tag (Figure 20) (FLAG is a commercial epitope for which reagents are available through IBI-Kodak). In the latter case this was achieved by the design of a synthetic oligonucleotide linker with an initiation site, antibody recognition sequence, and enterokinase cleavage site. Proteins were expressed under the control of either the pLac (eg Bluescript KS vector from Stratagene, LaJolla, CA.) or λpL (Shatman, et al., N.Y. Acad. Sci., 478: 233-248 (1986)) promoters and the radiophotoaffinity probe [Compound IV] shown to specifically crosslink proteins of the expected sizes in cell lysates. Lysates also contain Compound IA specific binding activity. One can conclude that both CSBP1 and CSBP2 are the molecular targets of the CSAIDs within cells.

Protein expressed in E. coli was purified by passage over an affinity matrix containing a monoclonal antibody to the FLAG epitope according to manufacturer's instructions.

Expression in Yeast

An alternative system for expression of CSBP is Saccharomyces cerevisiae, not only for purfication but also to assess function. The yeast *HOG1* (High Osmolarity Glycerol response) gene, (Brewster et al., surpa) encodes a MAP kinase which is a close homologue of CSBP. Mutant hog1D strains show reduced growth on high-osmolarity medium and functional complementation of this phenotype with CSBP was tested.

CSBP2 was engineered for yeast expression as follows. A XhoI site was introduced at the initiation codon of CSBP2 by the polymerase chain reaction (Mullis, and Faloona, Method in Enzymd., 155: 335-50 (1987) using the following oligonucleotide primers: 5'-cgcccteagatgtctcaggagaggcccacg-3' Seq. I.D. No. 13 and 3'-ctaagacctaaaacctgaccg-5', Seq. No. 14. The 525-bp PCR fragment was digested with XhoI and BglII and subcloned into the same sites in p138NBU, a modification of p138NB (McHale et al., Mol. Pharm. 39: 109-113 (1991) in which the TRP1 selectable marker was replaced with URA 3. The resulting plasmid was then digested with BglII and SalI and ligated with a BglII XhoII fragment containing the 3' end of CSBP2. The final construct contains partial 2micron sequences for maintenance at high copy number, with CSBP2 mRNA expression

driven by the copper-inducible CUP1 promoter and terminated by the yeast CYC1 transcriptional terminator. Plasmid p138NBU-CSBPN13B was found encode the wild-type CSBP2 protein. Transformations of parent (YPH499 MATa ura3-52 lys2-801^{am} ade2-101 trp1-D63 his3D200 leu2-D1) and hog1D (JBY10 [YPH499 + hog1::TRP1]) strains (Brewster, et al., J. Bacteriol. 153: 163-168 (1983) Ura$^+$ prototrophs were isolated and grown to A_{540} of 1.0 in synthetic complete medium lacking uracil (Hicks et al., Genetics 83: 245 (1976). CSBP2 expression was induced by the addition of 150 mM $CuSO_4$: Cells were harvested at 5 hr, resuspended 20 mM Tris-HCl pH7, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride and disrupted by vortexing in the presence of 0.45 mm glass beads. Extracts were centrifuged at 1,500 x g for 5 min at 4°.

Radiophotoaffinity probe (Compound IV) was shown to specifically crosslink a protein of the expected size in lysates of both p138NBU-CSBPN13A and p138NBU-CSBPN13B, which was not present in wild type or hog1D strains containing control plasmid (p138NBU) and grown under similar conditions. Lysates also contained ^{3}H Compound Ia specific binding activity, Therefore both CSB1 and CSB2 bind CSAIDS.

The proteins of this invention are preferably made by recombinant genetic engineering techniques. The isolated nucleic acids particularly the DNAs can be introduced into expression vectors by operatively linking the DNA to the necessary expression control regions (e.g. regulatory regions) required for gene expression. The vectors can be introduced into the appropriate host cells such as prokaryotic (e.g., bacterial), or eukaryotic (e.g., yeast or mammalian) cells by methods well known in the art (Ausubel et al.,supra).The coding sequences for the desired proteins having been prepared or isolated, can be cloned into any suitable vector or replicon. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. Examples of recombinant DNA vectors for cloning and host cells which they can transform include the bacteriophage λ (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), a baculovirus insect cell system, , YCp19 (Saccharomyces). See, generally, "DNA Cloning": Vols. I & II, Glover et al., eds. IRL Press Oxford (1985) (1987) and; T. Maniatis et al. "Molecular Cloning", Cold Spring Harbor Laboratory (1982).

The gene can be placed under the control of a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator (collectively referred to herein as "control" elements), so that the DNA sequence encoding the desired protein is transcribed into RNA in the host cell transformed by a vector containing this expression construction. The coding sequence may or may not contain a signal peptide or leader sequence. The subunit antigens of the present invention can be expressed using, for example, the E. coli tac promoter or the protein A gene (spa) promoter and signal sequence. Leader sequences can be removed by the bacterial host in post-translational processing. See, e.g., U.S. Patent Nos. 4,431,739; 4,425,437; 4,338,397.

In addition to control sequences, it may be desirable to add regulatory sequences which allow for regulation of the expression of the protein sequences relative to the growth of the host cell. Regulatory sequences are known to those of skill in the art, and examples include those which cause the expression of a gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Other types of regulatory elements may also be present in the vector, for example, enhancer sequences.

An expression vector is constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences (i.e., RNA polymerase which binds to the DNA molecule at the control sequences transcribes the coding sequence). Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; i.e., to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector, such as the cloning vectors described above. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site.

In some cases, it may be desirable to add sequences which cause the secretion of the polypeptide from the host organism, with subsequent cleavage of the secretory signal. Alternatively, gene fusions may be created whereby the gene encoding the binding protein of interested is fused to a gene encoding a product with other desirable properties. For example, a fusion partner could provide known assayable

activity (e.g. enzymatic) which could be used as an alternative means of selecting the binding protein. The fusion partner could be a structural element, such as a cell surface element such that the binding protein (a normally cytosolic component) could be displayed on the cell surfacte in the form of a fusion protein. It may also be desirable to produce mutants or analogs of the protein of interest. Mutants or analogs may be prepared by the deletion of a portion of the sequence encoding the protein, by insertion of a sequence, and/or by substitution of one or more nucleotides within the sequence. Techniques for modifying nucleotide sequences, such as site-directed mutagenesis and the formation of fusion proteins, are well known to those skilled in the art. See, e.g., T. Maniatis et al., supra; DNA Cloning, Vols. I and II, supra; Nucleic Acid Hybridization, supra.

A number of prokaryotic expression vectors are known in the art. See, e.g., U.S. Patent Nos. 4,578,355; 4,440,859; 4,436,815; 4,431,740; 4,431,739; 4,428,941; 4,425,437; 4,418,149; 4,411,994; 4,366,246; 4,342,832; see also U.K. Patent Applications GB 2,121,054; GB 2,008,123; GB 2,007,675; and European Patent Application 103,395. Yeast expression vectors are also known in the art. See, e.g., U.S. Patent Nos. 4,446,235; 4,443,539; 4,430,428; see also European Patent Applications 103,409; 100,561; 96,491. pSV2neo (as described in J. Mol. Appl. Genet. 1:327-341) which uses the SV40 late promoter to drive expression in mammalian cells or pCDNA1neo, a vector derived from pCDNA1(Mol. Cell Biol. 7:4125-29) which uses the CMV promoter to drive expression. Both these latter two vectors can be employed for transient or stable(e.g. using G418 or hygromycin resistance) expression in mammalian cells. Insect cell expression systems, e.g., Drosophila, are also useful, see for example, PCT applications US 89/05155 and US 91/06838 as well as EP application 88/304093.3 and Baculovirus expression systems.

Depending on the expression system and host selected, the proteins of the present invention are produced by growing host cells transformed by an expression vector described above under conditions whereby the protein of interest is expressed. The protein is then isolated from the host cells and purified. If the expression system secretes the protein into growth media, the protein can be purified directly from the media. If the protein is not secreted, it is isolated from cell lysates or recovered from the cell membrane fraction. The selection of the appropriate growth conditions and recovery methods are within the skill of the art.

An alternative method to identify proteins of the present invention is by constructing gene libraries, using the resulting clones to transform E. coli and pooling

and screening individual colonies using polyclonal serum or monocl nal antibodies to the desired binding protein.

The proteins of the present invention may also be produced by chemical synthesis such as solid phase peptide synthesis, using known amino acid sequences or amino acid sequences derived from the DNA sequence of the genes of interest. Such methods are known to those skilled in the art. Chemical synthesis of peptides is not particularly preferred.

The binding proteins of the present invention or their fragments comprising at least one epitope can be used to produce antibodies, both polyclonal and monoclonal. If polyclonal antibodies are desired, a selected mammal, (e.g., mouse, rabbit, goat, horse, etc.) is immunized with a binding protein of the present invention, or its fragment, or a mutated binding protein. Serum from the immunized animal is collected and treated according to known procedures. When serum containing polyclonal antibodies is used, the polyclonal antibodies can be purified by immunoaffinity chromatography or other known procedures.

Monoclonal antibodies to the proteins of the present invention, and to the fragments thereof, can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by using hybridoma technology is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. See, e.g., M. Schreier et al., "Hybridoma Techniques" (1980); Hammerling et al., "Monoclonal Antibodies and T-cell Hybridomas" (1981); Kennett et al., "Monoclonal Antibodies" (1980); see also U.S. Patent Nos. 4,341,761; 4,399,121; 4,427,783; 4,444,887; 4,452,570; 4,466,917; 4,472,500; 4,491,632; and 4,493,890. Panels of monoclonal antibodies produced against the protein of interest, or fragment thereof, can be screened for various properties; i.e., for isotype, epitope, affinity, etc. Alternatively, genes encoding the monoclonals of interest may be isolated from the hybridomas by PCR techniques known in the art and cloned and expressed in the appropriate vectors. Monoclonal antibodies are useful in purification, using immunoaffinity techniques, of the individual proteins against which they are directed. The antibodies of this invention, whether polyclonal or monoclonal have additional utility in that they may be employed reagents in immunoassays, RIA, ELISA, and the like. In addition they can be used to isolate the CSBP from human cells and determine the effect of different stimuli and compounds on the phosphorylation state and protein kinase activity of endogenous CSBP. The antibodies could be used to establish a tissue culture based assay for

discovery or modification of novel compounds which block the phosphorylation or kinase activity of CSBP. An example of such an assay would be to incubate human monocytes or monocytic cell lines with a compound or compound mixture prior to treatment with LPS for a defined time period, followed by immunoprecipitation of CSBP with antibody and assessment of its phosphorylation state via immunoblot or chromatography or measurement of its kinase activity with appropriate protein or peptide substrate.

This invention provides a method for determining whether a ligand previously not known to bind to a CSBP can bind to such a protein. The method comprises contacting the ligand to be identified with cytosolic fraction from THP.1 cells and measuring its ability to compete with a known radioactive CSAID, as described above, in a CSAIDs binding assay. Alternative methods include contacting the ligand to be identified with a whole-cell expressing the coding sequence of a CSBP under conditions sufficient for binding of ligands previously identified as binding to such a receptor. In other embodiments cell membrane fractions comprising the CSBP fusions or isolated CSBP free or immobilized on solid supports may be used to measure binding of the ligand to be tested. When recombinant cells are used for purposes of expression of the CSBP it is preferred to use cells with little or no endogenous CSBP activity so that binding if any is due to the presence of the expressed protein of interest. As mentioned previously, a specifically designed indicator of receptor binding can be constructed. For example a fusion protein can be made by fusing the CSBP of this invention with a protein domain which is sensitive to CSBP/ ligand binding. Such a domain referred to here as an indicator domain is capable, itself, or in association with accessory molecules, of generating an analytically detectable signal which is indicative of receptor ligand binding. A variation of this approach is to express CSBP as a fusion protein (e.g., fused to FLAG peptide) in THP.1 or other mammalian cells, and to use the fusion peptide as a means of isolating the recombinant CSBP after suitable stimulation and pretreatment of THP.1 cells. Such expression can be achieved with numerous mammalian expression vectors which utilize viral promoters, eg CMV, RSV and polyadenylation sequences, et. SV40, bovine growth hormone, and a selectable marker such as G418 or hygromycin for selection of stable transfectants.

Cytosolic preparations from transfected or transformed cells expressing such fusions may be employed. All of the above techniques that are useful for ligand identification are also useful in drug screening and drug development protocols.

Alternatively, the purified recombinant protein could be used to substitute for crude THP.1 cell lysates in a competitive binding assay with Compound Ia. This assay is useful to screen for novel compound which bind CSBP, or as a way to assess alterations to compound which is known to bind. The availability of purified protein allows alternative configurations of the assay from those described previously for the crude material. For example, if the protein is covalently linked to a tag, such a protein binding site for configuration in a colorimetic assay, e.g., conjugated antibody, or to an enzyme for direct detection of enzyme activity, e.g., horseradish peroxidase or alkaline phosphatase, binding to novel compounds displayed on a solid matrix could be detected. Such compounds could include low molecular weight organic molecules, peptides, peptoids, and proteins. In the latter case, the protein can be used as a way to isolate other proteins in its signaling cascade, for example, those that are in the pathway for activation of cytokine translation in activated monocytes. The protein may also be used to isolate naturally occurring regulatory molecules within mammalian cells that act by a CSAIDs binding mechanism. Finally, the protein can be used to identify target peptides displayed on the surface of phage.

The knowledge that the CSBPs encode protein kinases suggest that recombinant forms can be used to establish a protein kinase activity. Typically this would involve the direct incubation of CSBP with a protein or peptide substrate in the presence of γ-^{32}P- ATP, followed by the measurement of radioactivity incorporated into the substrate by separation and counting. Separation methods include immunoprecipitation, conjugation of substrate to a bead allowing separation by centrifugation or determination of incorporation by scintillation proximity assay, SDS-PAGE followed by autoradiography or biosensor analysis. While the specific substrates are not yet known, candidates include CSBP itself (autophosphorylation) and peptides related to known MAP kinase substrates. Other substances might be discovered by incubating CSBP with random peptides conjugated to solid supports or displayed by phage (see above) or by incubation of CSBP with mammalian cell lysates (e.g. THP.1 cell lysates) and γ-^{32}P- ATP, followed by separation of the labelled target proteins, and sequencing. Kinase activity may also be detected by use of antiphosphotyrosine antibodies. The protein kinase activity of CSBP may require incubation with a specific MEK. This may be achieved by preincubating CSBP with lysates from stimulated eukaryotic cells (e.g., LPS treated THP.1 cells) and ATP. Alternatively, it may be possible to isolate a more active form of CSBP from HOG1 deletion strains of yeast expressing the human CSBP and grown in high osmolarity conditions.

These assays permit the discovery and modification f compounds which inhibit CSBP kinase activity in vitro. Such compounds would be expected to block cytokine synthesis in a comparable fashion to the compounds described herein. They could also lead to the discovery of novel substrates which themselves may be viable targets for discovery of novel compounds which block cytokine production.

It is expected that CSBPs, like other MAP kinases, will be activated by a MEK, hence the recombinant protein would allow the establishment of a second assay which measures the ability of CSBP to be phosphorylated by putative MEKs. In this case fractions from stimulated cell lysates (eg THP.1 cells stimulated with LPS) are incubated with CSBP in the presence of γ-^{32}P-ATP, and the incorporation of ^{32}P-label into CSBP measured by separation and counting. Separation can be achieved in a number of ways: one way is to use a CSBP fused to an peptide or protein and separate via affinity chromatography or immunoprecipitation with the peptide or protein directed antibody. Alternatively the CSBP can be directly conjugated to beads or bound through a fusion peptide or protein (e.g., FLAG (peptide), glutathionine-S-transferase) and separated by centrifugation after incubation with cell lysates. Also tyrosine phosphorylation of CSBP could be detected by immunoprecipitation or immunoblot with commercially available anti-phosphotyrosine antibodies.

These assays can be used to discover compounds which block the activation of CSBP protein kinase activity and to improve the potency of already discovered compounds. These compounds would be expected to have utility due to their blocking of cytokine synthesis. The assays are also useful to discover novel MEKs which themselves may become targets for novel compounds which would block cytokine synthesis.

The ability of human CSBP to rescue a HOG1 deletion strain upon growth in conditions of high osmolarity allows for the direct screening of compounds which block CSBP activity in vivo. For example, compounds could be screened for their ability to block growth of a CSBP +/HOG1- yeast strain in high osmolarity but which have no effect on growth of the same strain in standard osmolarity or on a CSBP-/HOG1+ in high osmolarity. The sensitivity of the yeast based assay can be increased by introducing host mutations that affect the cell membrane and permeability (Gaber, et al., Mol. Cell. Biol. 9: 3447-3456. (1989).

In a compound screening embodiment of this invention, the CSBP in isolated, immobilized or cell bound form is contacted with a plurality of candidate molecules and those candidates are selected which bind to and interact with the protein. The

binding or interaction can be measured directly by using radioactively labeled candidate of interest or indirectly by measuring an effect resulting from the interaction or binding of the candidate compound. Alternatively, the candidate compounds can be subjected to a competition screening assays, in which a known ligand, preferably labeled with an analytically detectable reagent, most notably radioactivity, is introduced with the compounds to be tested and the compound's capacity to inhibit or enhance the binding of the labeled ligand is measured. Compounds are screened for their increased affinity and selectivity for the CSBP.

To illustrate this aspect of the invention a natural product screen was performed.

The standard assay in which bound ligand is separated from free by exclusion chromatography using mini-columns was used to initiate a screening effort. Approximately 625 marine extracts, 202 microbial extracts and 233 extracts of plant material were tested for inhibition of ^{3}H-Compound I binding to THP.1 cytosol. Two extracts were confirmed as antagonists of this binding, with IC_{50}'s of around 200 and 80 µg/ml respectively. This low hit-rate (0.2%) coupled with the failure to observe inhibition by any of a selected group of "nuisance extracts" indicates that the assay is sufficiently selective and robust to support a screening effort. While the potency of these two hits is rather weak, they were nevertheless accepted as leads for isolation of their active principle so that the primary assay could be evaluated as well as identification of the bioactive compounds.

The two extracts were subsequently fractionated and characterized.

Further refinement of the binding assay to facilitate high throughout screening can be achieved by the minor modification of separating bound ligand from free ligand using spin columns.

This invention also contemplates pharmaceutical compositions comprising compounds when identified by the above methods and a pharmaceutically acceptable carrier. Pharmaceutical compositions of proteineous drugs of this invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The compositions for parenteral administration will commonly comprise a solution of the compounds of the invention or a cocktail thereof dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, e.g., water, buffered water, 0.4% saline, 0.3% glycine, and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as

required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the compound of the inventi n in such pharmaceutical formulation can very widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and 50 mg of a compound of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain 250 ml of sterile Ringer's solution, and 150 mg of a compound of the invention. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania.

The compounds described herein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. This technique has been shown to be effective with conventional proteins and art-known lyophilization and reconstitution techniques can be employed.

In situations where the identified drug is non-proteineous, it may be administered alone or in combinantion with pharmaceutically acceptable carriers. The proportion of which is determined by the solubility and chemical nature of the compound, chosen route of administration and standard pharmaceutical practice. For example, they may be administered orally in the form of tablets or capsules containing such excipients as starch, milk sugar, certain types of clay and so forth. They may be administered sublingually in the form of troches or lozenges in which the active ingredient is mixed with sugar and corn syrups, flavoring agents and dyes; and then dehydrated sufficiently to make it suitable for pressing into a solid form. They may be administered orally in the form of solutions which may be injected parenterally, that is, intramuscularly, intravenously or subcutaneously. For parenteral administration they may be used in the form of a sterile solution containing other solutes, for example, enough saline or glucose to make the solution isotonic.

The physician will determine the dosage of the present therapeutic agents which will be most suitable and it will vary with the form of administration and the particular compound chosen, and furthermore, it will vary with the particular patient under treatment. He will generally wish to initiate treatment with small dosages substantially less than the optimum dose of the compound and increase the dosage by

small increments until the optimum effect under the circumstances is reached. It will generally be found that when the composition is administered orally, larger quantities of the active agent will be required to produce the same effect as a smaller quantity given parenterally. The compounds are useful in the same manner as ther serotonergic agents and the dosage level is of the same order of magnitude as is generally employed with these other therapeutic agents. The therapeutic dosage will generally be from 1 to 10 milligrams per day and higher although it may be administered in several different dosage units. Tablets containing from 0.5 to 10 mg. of active agent are particularly useful.

Depending on the patient condition, the pharmaceutical composition of the invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease in an amount sufficient to cure or at least partially arrest the disease and its complications. In prophylactic applications, compositions containing the present compounds or a cocktail thereof are administered to a patient not already in a disease state to enhance the patient's resistance.

Single or multiple administrations of the pharmaceutical compositions can be carried out with dose levels and pattern being selected by the treating physician. In any event, the pharmaceutical composition of the invention should provide a quantity of the compounds of the invention sufficient to effectively treat the patient.

The nucleic acid embodiment of this invention is particularly useful in providing probes capable of specific hybridization with human CSBP sequences. Probing technology is well known in the art and it is appreciated that the size of the probes can vary widely but it is preferred that the probe be at least 15 nucleotides in length. It is also appreciated that such probes can be and are preferably labeled with an analytically detectable reagent to facilitate identification of the probe. Useful reagents include but are not limited to radioactivity, fluorescent dyes or enzymes capable of catalyzing the formation of a detectable product. This invention contemplates, for example using receptor encoding probes in the diagnostic evaluation of disease states characterized by an abnormal, i.e. increased or decreased level of receptor gene expression. Alternatively, the probes can be used to identify individuals carrying chromosomal or molecular mutations in the gene encoding the receptor. Depending on the conditions employed by the ordinary skilled artisan, the probes can be used to identify and recover additional examples of this receptor (in its genomic or cDNA form) from other cell types and individuals. As a general rule the

more stringent the hybridization conditions the more cl sely related genes will be that are recovered.

Also within the scope of this invention are antisense oligonucleotides predicated upon the sequences disclosed herein for the CSBP. Synthetic oligonucleotides or related antisense chemical structural analogs are designed to recognize and specifically bind to a target nucleic acid encoding the receptor gene and inhibit gene expression, e.g., the translation of the gene when the target nucleic acid is mRNA. Although not wishing to be bound to a particular theory for the mechanism of action of antisense drugs, it is believed that such drugs can act by one or more of the following mechanisms: by binding to mRNA and inducing degradation by endogenous nucleases such as RNase I or by inhibiting the translation of mRNA by inhibiting its binding to regulatory factors or ribosomal components necessary for productive protein synthesis. Additionally the antisense sequences can be use as components of a complex macromolecular arrays in which the sequences are combined with ribozyme sequences or reactive chemical groups and are used to specifically target mRNAs of interest and degrade or chemically modify said mRNAs. The general field of antisense technology is illustrated by the following disclosures which are incorporated herein by reference for purposes of background (Cohen, J.S., Trends in Pharm. Sci. 10:435(1989) and Weintraub, H.M. Scientific American Jan.(1990) at page 40).

This invention also contemplates the use of the DNA sequences disclosed herein in gene therapy. Because CSBP is a protein kinase it is possible to make a site specific mutant which is inactive as a kinase but will block activation of the endogenous CSBP when coexpressed in the same cell, i.e., it is a dominant negative mutant (Kolch et al., Nature 349: 426-428 (1991). The DNA encoding this mutant protein could be used in gene therapy to reduce chronic inflammation. There are many vector and delivery systems available to direct DNA into target cells in vivo, e.g. adenovirus, retroviruses.

This invention also contemplates antibodies, monoclonal or polyclonal directed to epitopes corresponding to amino acid sequences disclosed herein from the CSBP. Particularly important regions of the receptor for immunological purposes are those regions associated with ligand binding domains of the protein. Antibodies directed to the regions are particularly useful in diagnostic and therapeutic applications because of their effect upon protein- ligand interaction. Methods for the production of polyclonal and monoclonal antibodies are well known, see for example Chap. 11 of Ausubel et al. (supra).

This invention also provides pharmaceutical compositions comprising an effective amount of antibody or fragment thereof directed against the CSBP to block binding of the naturally occurring ligands to that protein in order to treat or ameliorate disease states associated with protein activation.

Transgenic, non-human, animals may be obtained by transfecting appropriate fertilized eggs or embryos of a host with nucleic acids encoding the CSBP disclosed herein, see for example U.S.Patents 4,736,866; 5,175,385; 5,175,384 and 5,175,386. The resultant transgenic animal may be used as a model for the study of CSBP/ligand interaction. Particularly, useful transgenic animals are those which display a detectable phenotype associated with the expression of the protein. Drugs may then be screened for their ability to reverse or exacerbate the relevant phenotype. This invention also contemplates operatively linking the CSBP coding gene to regulatory elements which are differentially responsive to various temperature or metabolic conditions, thereby effectively turning on or off the phenotypic expression in response to those conditions.

The nucleic acid probes disclosed herein can be used to clone the cognate version of the human CSBP gene from a desired experimental animal species; for example the murine version. Strains of mice can be developed in which said gene has been eliminated by conventional gene knock-out technology. The gene can then be substituted/or replaced by the human CSBP DNA of this invention to yield a mouse for screening candidate drugs in vivo. Similar gene knockout and human protein inhibition studies can also be performed with yeast.

The purified protein of this invention is also useful in a reagent for structural studies with and without bound drug candidates as a means for the rational design of novel drugs affecting CSBP. For example, the recombinant protein may be used to derive the structure of the protein alone or complexed with Compound Ia and related compounds through X-ray crustallography, NMR or modelling from published structures of related protein kinases, e.g., CSK. A structure fosters an understanding of how the inhibitory compounds bind, and can lead to the design or discovery of further compounds which can block CSBP activity and hence be inhibitors of cytokine synthesis. There are now several examples of such structure-based design for other protein targets, e.g., HIV protease. Given the similarity of CSBP to several other kinases (e.g. the MAP and CDC kinases), such structural information will be useful in designing novel compounds which inhibit other members of the kinase family.

SEQUENCE LISTING

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(iii) NUMBER OF SEQUENCES: 16

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: US 08/123,175
(B) FILING DATE: 17-SEP-1993

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Monocyte
(H) CELL LINE: THP.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile Thr Ala Ala Gln Ala Leu Ala His Ala Tyr Phe Ala Gln Tyr
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(G) CELL TYPE: MONOCYTE
(H) CELL LINE: THP.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Gln Leu Leu Asn Asn Ile Val Lys Phe Gln Lys Leu Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(G) CELL TYPE: MONOCYTE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCYCAYGCTA YTTYGCYCAR TA 22

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(G) CELL TYPE: MONOCYTE

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AAYAAYATYK TBAARTTYCA AA 22

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(G) CELL TYPE: MONOCYTE
(H) CELL LINE: THP.1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Ile Val Lys Cys Gln Lys Leu Thr
1 5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 285 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(G) CELL TYPE: MONOCYTE
(H) CELL LINE: THP.1

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..285

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
AAC ATT GTG AAA TGT CAG AAG CTT ACA GAT GAC CAT GTT CAG TTC CTT      48
Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln Phe Leu
 1               5                  10                  15

ATC TAC CAA ATT CTC CGA GGT CTA AAG TAT ATA CAT TCA GCT GAC ATA      96
Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asp Ile
            20                  25                  30

ATT CAC AGG GAC CTA AAA CCT AGT AAT CTA GCT GTG AAT GAA GAC TGT     144
Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu Asp Cys
        35                  40                  45

GAG CTG AAG ATT CTG GAT TTT GGA CTG GCT CGG CAC ACA GAT GAT GAA     192
Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp Asp Glu
    50                  55                  60

ATG ACA GGC TAC GTG GCC ACT AGG TGG TAC AGG GCT CCT GAG ATC ATG     240
Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met
65                  70                  75                  80

CTG AAC TGG ATG CAT TAC AAC CAG ACA GGT GGT ATT TGG GTC AAG         285
Leu Asn Trp Met His Tyr Asn Gln Thr Gly Gly Ile Trp Val Lys
                85                  90                  95
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln Phe Leu
 1               5                  10                  15
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Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asp Ile
20 25 30

Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu Asp Cys
35 40 45

Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp Asp Glu
50 55 60

Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met
65 70 75 80

Leu Asn Trp Met His Tyr Asn Gln Thr Gly Gly Ile Trp Val Lys
85 90 95

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HOMO SAPIENS
(G) CELL TYPE: MONOCYTE
(H) CELL LINE: THP.1

(ix) FEATURE:
(A) NAME/KEY: 3'UTR
(B) LOCATION: 1..392

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAAGTCCCAA TCCTCCCCAA CCACAGCAAG TTGAATTTAT CAACCATGTT GGGTTGTAAA 60

TGCTCGTGTG ATTTCCTACA AGAAATACCT GCTCTGAATA TTTTTGTAAT AAAGGTCTTT 120

GCACATGTGA CCCACAATAC GTGTTAGGAG CCTGCATGCT CTGGAAGCCT GGACTCTAAG 180

CTGGAGCTCT TGGAAGAGCT CTTCGGTTTC TGAGCATAAT GCTCCCATCT CCTGATTTCT 240

CTGAACAGAA AACAAAAGAG AGAATGAGGG AAATTGCTAT TTTATTTGTA TTGATGAACT 300

TGGCTGTAAT CAGTTATGCC GTATAGGATG TCAGACAATA CCACTGGTTA AAATAAAGCC 360

TATTTTTCAA ATTTAAAAAA AAAAAAAAAA AA 392

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Monocyte

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCTCGGAGAA TTTGGTAGAT AAGG 24

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Monocyte

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AACATTGTGA AATGTCAGAA GCTTACAGAT GACCAT 36

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3813 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Monocyte

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 379..1461

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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CCTCCTGGTA TAATCTGGAA CCGCGACCAC TGGAGCCTTA GCGGGCGCAG CAGCTGGAAC   60

GGGAGTACTG CGACGCAGCC CGGAGTCGGC CTTGTAGGGG CGAAGGTGCA GGGAGATCGC  120

GGCGGGCGCA GTCTTGAGCG CCGGAGCGCG TCCCTGCCCT TAGCGGGGCT TGCCCCAGTC  180

GCAGGGGCAC ATCCAGCCGC TGCGGCTGAC AGCAGCCGCG CGCGCGGGAG TCTGCGGGGT  240

CGCGGCAGCC GCACCTGCGC GGGCGACCAG CGCAAGGTCC CCGCCCGGCT GGGCGGGCAG  300

CAAGGGCCGG GGAGAGGGTG CGGGTGCAGG CGGGGGCCCC ACAGGGCCAC CTTCTTGCCC  360

GGCGGCTGCC GCTGGAAA ATG TCT CAG GAG AGG CCC ACG TTC TAC CGG CAG      411
                   Met Ser Gln Glu Arg Pro Thr Phe Tyr Arg Gln
                     1               5                  10

GAG CTG AAC AAG ACA ATC TGG GAG GTG CCC GAG CGT TAC CAG AAC CTG      459
Glu Leu Asn Lys Thr Ile Trp Glu Val Pro Glu Arg Tyr Gln Asn Leu
             15                  20                  25

TCT CCA GTG GGC TCT GGC GCC TAT GGC TCT GTG TGT GCT GCT TTT GAC      507
Ser Pro Val Gly Ser Gly Ala Tyr Gly Ser Val Cys Ala Ala Phe Asp
         30                  35                  40

ACA AAA ACG GGG TTA CGT GTG GCA GTG AAG AAG CTC TCC AGA CCA TTT      555
Thr Lys Thr Gly Leu Arg Val Ala Val Lys Lys Leu Ser Arg Pro Phe
     45                  50                  55

CAG TCC ATC ATT CAT GCG AAA AGA ACC TAC AGA GAA CTG CGG TTA CTT      603
Gln Ser Ile Ile His Ala Lys Arg Thr Tyr Arg Glu Leu Arg Leu Leu
 60                  65                  70                  75
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AAA CAT ATG AAA CAT GAA AAT GTG ATT GGT CTG TTG GAC GTT TTT ACA      651
Lys His Met Lys His Glu Asn Val Ile Gly Leu Leu Asp Val Phe Thr
                80                  85                  90

CCT GCA AGG TCT CTG GAG GAA TTC AAT GAT GTG TAT CTG GTG ACC CAT      699
Pro Ala Arg Ser Leu Glu Glu Phe Asn Asp Val Tyr Leu Val Thr His
            95                  100                 105

CTC ATG GGG GCA GAT CTG AAC AAC ATT GTG AAA TGT CAG AAG CTT ACA      747
Leu Met Gly Ala Asp Leu Asn Asn Ile Val Lys Cys Gln Lys Leu Thr
        110                 115                 120

GAT GAC CAT GTT CAG TTC CTT ATC TAC CAA ATT CTC CGA GGT CTA AAG      795
Asp Asp His Val Gln Phe Leu Ile Tyr Gln Ile Leu Arg Gly Leu Lys
    125                 130                 135

TAT ATA CAT TCA GCT GAC ATA ATT CAC AGG GAC CTA AAA CCT AGT AAT      843
Tyr Ile His Ser Ala Asp Ile Ile His Arg Asp Leu Lys Pro Ser Asn
140                 145                 150                 155

CTA GCT GTG AAT GAA GAC TGT GAG CTG AAG ATT CTG GAT TTT GGA CTG      891
Leu Ala Val Asn Glu Asp Cys Glu Leu Lys Ile Leu Asp Phe Gly Leu
                160                 165                 170

GCT CGG CAC ACA GAT GAT GAA ATG ACA GGC TAC GTG GCC ACT AGG TGG      939
Ala Arg His Thr Asp Asp Glu Met Thr Gly Tyr Val Ala Thr Arg Trp
            175                 180                 185

TAC AGG GCT CCT GAG ATC ATG CTG AAC TGG ATG CAT TAC AAC CAG ACA      987
Tyr Arg Ala Pro Glu Ile Met Leu Asn Trp Met His Tyr Asn Gln Thr
        190                 195                 200

GTT GAT ATT TGG TCA GTG GGA TGC ATA ATG GCC GAG CTG TTG ACT GGA     1035
Val Asp Ile Trp Ser Val Gly Cys Ile Met Ala Glu Leu Leu Thr Gly
    205                 210                 215

AGA ACA TTG TTT CCT GGT ACA GAC CAT ATT AAC CAG CTT CAG CAG ATT     1083
Arg Thr Leu Phe Pro Gly Thr Asp His Ile Asn Gln Leu Gln Gln Ile
220                 225                 230                 235
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ATG CGT CTG ACA GGA ACA CCC CCC GCT TAT CTC ATT AAC AGG ATG CCA      1131
Met Arg Leu Thr Gly Thr Pro Pro Ala Tyr Leu Ile Asn Arg Met Pro
                240                 245                 250

AGC CAT GAG GCA AGA AAC TAT ATT CAG TCT TTG ACT CAG ATG CCG AAG      1179
Ser His Glu Ala Arg Asn Tyr Ile Gln Ser Leu Thr Gln Met Pro Lys
            255                 260                 265

ATG AAC TTT GCG AAT GTA TTT ATT GGT GCC AAT CCC CTG GCT GTC GAC      1227
Met Asn Phe Ala Asn Val Phe Ile Gly Ala Asn Pro Leu Ala Val Asp
        270                 275                 280

TTG CTG GAG AAG ATG CTT GTA TTG GAC TCA GAT AAG AGA ATT ACA GCG      1275
Leu Leu Glu Lys Met Leu Val Leu Asp Ser Asp Lys Arg Ile Thr Ala
    285                 290                 295

GCC CAA GCC CTT GCA CAT GCC TAC TTT GCT CAG TAC CAC GAT CCT GAT      1323
Ala Gln Ala Leu Ala His Ala Tyr Phe Ala Gln Tyr His Asp Pro Asp
300                 305                 310                 315

GAT GAA CCA GTG GCC GAT CCT TAT GAT CAG TCC TTT GAA AGC AGG GAC      1371
Asp Glu Pro Val Ala Asp Pro Tyr Asp Gln Ser Phe Glu Ser Arg Asp
                320                 325                 330

CTC CTT ATA GAT GAG TGG AAA AGC CTG ACC TAT GAT GAA GTC ATC AGC      1419
Leu Leu Ile Asp Glu Trp Lys Ser Leu Thr Tyr Asp Glu Val Ile Ser
            335                 340                 345

TTT GTG CCA CCA CCC CTT GAC CAA GAA GAG ATG GAG TCC TGAGCACCTG       1468
Phe Val Pro Pro Pro Leu Asp Gln Glu Glu Met Glu Ser
        350                 355                 360

GTTTCTGTTC TGTTGATCCC ACTTCACTGT GAGGGGAAGG CCTTTTCACG GGAACTCTCC    1528

AAATATTATT CAAGTGCCTC TTGTTGCAGA GATTTCCTCC ATGGTGGAAG GGGGTGTGCG    1588

TGCGTGTGCG TGCGTGTTAG TGTGTGTGCA TGTGTGTGTC TGTCTTTGTG GGAGGGTAAG    1648

ACAATATGAA CAAACTATGA TCACAGTGAC TTTACAGGAG GTTGTGGATG CTCCAGGGCA    1708

GCCTCCACCT TGCTCTTCTT TCTGAGAGTT GGCTCAGGCA GACAAGAGCT GCTGTCCTTT    1768
```

TAGGAATATG TTCAATGCAA AGTAAAAAAA TATGAATTGT CCCCAATCCC GGTCATGCTT 1828

TTGCCACTTT GGCTTCTCCT GTGACCCCAC CTTGACGGTG GGGCGTAGAC TTGACAACAT 1888

CCCACAGTGG CACGGAGAGA AGGCCCATAC CTTCTGGTTG CTTCAGACCT GACACCGTCC 1948

CTCAGTGATA CGTACAGCCA AAAAGGACCA ACTGGCTTCT GTGCACTAGC CTGTGATTAA 2008

CTTGCTTAGT ATGGTTCTCA GATCTTGACA GTATATTTGA AACTGTAAAT ATGTTTGTGC 2068

CTTAAAAGGA GAGAAGAAAG TGTAGATAGT TAAAAGACTG CAGCTGCTGA AGTTCTGAGC 2128

CGGGCAAGTC GAGAGGGCTG TTGGACAGCT GCTTGTGGGC CCGGAGTAAT CAGGCAGCCT 2188

TCATAGGCGG TCATGTGTGC ATGTGAGCAC ATGCGTATAT GTGCGTCTCT CTTTCTCCCT 2248

CACCCCCAGG TGTTGCCATT TCTCTGCTTA CCCTTCACCT TTGGTGCAGA GGTTTCTTGA 2308

ATATCTGCCC CAGTAGTCAG AAGCAGGTTC TTGATGTCAT GTACTTCCTG TGTACTCTTT 2368

ATTTCTAGCA GAGTGAGGAT GTGTTTTGCA CGTCTTGCTA TTTGAGCATG CACAGCTGCT 2428

TGTCCTGCTC TCTTCAGGAG GCCCTGGTGT CAGGCAGGTT TGCCAGTGAA GACTTCTTGG 2488

GTAGTTTAGA TCCCATGTCA CCTCAGCTGA TATTATGGCA AGTGATATCA CCTCTCTTCA 2548

GCCCCTAGTG CTATTCTGTG TTGAACACAA TTGATACTTC AGGTGCTTTT GATGTGAAAA 2608

TCATGAAAAG AGGAACAGGT GGATGTATAG CATTTTTATT CATGCCATCT GTTTTCAACC 2668

AACTATTTTT GAGGAATTAT CATGGGAAAA GACCAGGGCT TTTCCCAGGA ATATCCCAAA 2728

CTTCGGAAAC AAGTTATTCT CTTCACTCCC AATAACTAAT GCTAAGAAAT GCTGAAAATC 2788

AAAGTAAAAA ATTAAAGCCC ATAAGGCCAG AAACTCCTTT TGCTGTCTTT CTCTAAATAT 2848

GATTACTTTA AAATAAAAAA GTAACAAGGT GTCTTTTCCA CTCCTATGGA AAAGGGTCTT 2908

CTTGGCAGCT TAACATTGAC TTCTTGGTTT GGGGAGAAAT AAATTTTGTT TCAGAATTTT 2968

GTATATTGTA GGAATCCCTT TGAGAATGTG ATTCCTTTTG ATGGGGAGAA AGGGCAAATT 3028

ATTTTAATAT TTTGTATTTT CAACTTTATA AAGATAAAAT ATCCTCAGGG GTGGAGAAGT 3088

GTCGTTTTCA TAACTTGCTG AATTTCAGGC ATTTTGTTCT ACATGAGGAC TCATATATTT 3148

AAGCCTTTTG TGTAATAAGA AAGTATAAAG TCACTTCCAG TGTTGGCTGT GTGACAGAAT 3208

CTTGTATTTG GGCCAAGGTG TTTCCATTTC TCAATCAGTG CAGTGATACA TGTACTCCAG 3268

AGGGACGGGT GGACCCCCTG AGTCAACTGG AGCAAGAAGG AAGGAGGCAG ACTGATGGCG 3328

ATTCCCTCTC ACCCGGGACT CTCCCCCTTT CAAGGAAAGT GAACCTTTAA AGTAAAGGCC 3388

TCATCTCCTT TATTGCAGTT CAAATCCTCA CCATCCACAG CAAGATGAAT TTTATCAGCC 3448

ATGTTTGGTT GTAAATGCTC GTGTGATTTC CTACAGAAAT ACTGCTCTGA ATATTTTGTA 3508

ATAAAGGTCT TTGCACATGT GACCACATAC GTGTTAGGAG GCTGCATGCT CTGGAAGCCT 3568

GGACTCTAAG CTGGAGCTCT TGGAAGAGCT CTTCGGTTTC TGAGCATAAT GCTCCCATCT 3628

CCTGATTTCT CTGAACAGAA AACAAAAGAG AGAATGAGGG AAATTGCTAT TTTATTTGTA 3688

TTCATGAACT TGGCTGTAAT CAGTTATGCC GTATAGGATG TCAGACAATA CCACTGGTTA 3748

AAATAAAGCC TATTTTTCAA ATTTAAAAAA AAAAAAAAAA AAGTCCAGCA ATTTCGTTAC 3808

TTATG 3813

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Ser Gln Glu Arg Pro Thr Phe Tyr Arg Gln Glu Leu Asn Lys Thr
 1               5                  10                  15

Ile Trp Glu Val Pro Glu Arg Tyr Gln Asn Leu Ser Pro Val Gly Ser
            20                  25                  30

Gly Ala Tyr Gly Ser Val Cys Ala Ala Phe Asp Thr Lys Thr Gly Leu
        35                  40                  45

Arg Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Ser Ile Ile His
    50                  55                  60

Ala Lys Arg Thr Tyr Arg Glu Leu Arg Leu Leu Lys His Met Lys His
65                  70                  75                  80

Glu Asn Val Ile Gly Leu Leu Asp Val Phe Thr Pro Ala Arg Ser Leu
                85                  90                  95

Glu Glu Phe Asn Asp Val Tyr Leu Val Thr His Leu Met Gly Ala Asp
            100                 105                 110

Leu Asn Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln
        115                 120                 125

Phe Leu Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala
    130                 135                 140

Asp Ile Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu
145                 150                 155                 160
```

```
Asp Cys Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp
                165                 170                 175

Asp Glu Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu
            180                 185                 190

Ile Met Leu Asn Trp Met His Tyr Asn Gln Thr Val Asp Ile Trp Ser
        195                 200                 205

Val Gly Cys Ile Met Ala Glu Leu Leu Thr Gly Arg Thr Leu Phe Pro
    210                 215                 220

Gly Thr Asp His Ile Asn Gln Leu Gln Gln Ile Met Arg Leu Thr Gly
225                 230                 235                 240

Thr Pro Pro Ala Tyr Leu Ile Asn Arg Met Pro Ser His Glu Ala Arg
                245                 250                 255

Asn Tyr Ile Gln Ser Leu Thr Gln Met Pro Lys Met Asn Phe Ala Asn
            260                 265                 270

Val Phe Ile Gly Ala Asn Pro Leu Ala Val Asp Leu Leu Glu Lys Met
        275                 280                 285

Leu Val Leu Asp Ser Asp Lys Arg Ile Thr Ala Ala Gln Ala Leu Ala
    290                 295                 300

His Ala Tyr Phe Ala Gln Tyr His Asp Pro Asp Asp Glu Pro Val Ala
305                 310                 315                 320

Asp Pro Tyr Asp Gln Ser Phe Glu Ser Arg Asp Leu Leu Ile Asp Glu
                325                 330                 335

Trp Lys Ser Leu Thr Tyr Asp Glu Val Ile Ser Phe Val Pro Pro Pro
            340                 345                 350

Leu Asp Gln Glu Glu Met Glu Ser
        355                 360
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1423 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(G) CELL TYPE: Monocyte

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 227..1309

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
CCACTCCTGG TATAATCTCG CCCCAGTCGC AGGGGCACAT CCAGCCGCTG CGGCTGACAG    60

CAGCCGCGCG CGCGGGAGTC TGCGGGGTCG CGGCAGCCGC ACCTGCGCGG GCGACCAGCG   120

CAAGGTCCCC GCCCGGCTGG GCGGGCAGCA AGGGCCGGGG AGAGGGTGCG GGTGCAGGCG   180

GGGGCCCCAC AGGGCCACCT TCTTGCCCGG CGGCTGCCGC TGGAAA ATG TCT CAG       235
                                                      Met Ser Gln
                                                        1

GAG AGG CCC ACG TTC TAC CGG CAG GAG CTG AAC AAG ACA ATC TGG GAG      283
Glu Arg Pro Thr Phe Tyr Arg Gln Glu Leu Asn Lys Thr Ile Trp Glu
  5                  10                  15
```

```
GTG CCC GAG CGT TAC CAG AAC CTG TCT CCA GTG GGC TCT GGC GCC TAT     331
Val Pro Glu Arg Tyr Gln Asn Leu Ser Pro Val Gly Ser Gly Ala Tyr
 20                  25                  30                  35

GGC TCT GTG TGT GCT GCT TTT GAC ACA AAA ACG GGG TTA CGT GTG GCA     379
Gly Ser Val Cys Ala Ala Phe Asp Thr Lys Thr Gly Leu Arg Val Ala
                 40                  45                  50

GTG AAG AAG CTC TCC AGA CCA TTT CAG TCC ATC ATT CAT GCG AAA AGA     427
Val Lys Lys Leu Ser Arg Pro Phe Gln Ser Ile Ile His Ala Lys Arg
             55                  60                  65

ACC TAC AGA GAA CTG CGG TTA CTT AAA CAT ATG AAA CAT GAA AAT GTG     475
Thr Tyr Arg Glu Leu Arg Leu Leu Lys His Met Lys His Glu Asn Val
         70                  75                  80

ATT GGT CTG TTG GAC GTT TTT ACA CCT GCA AGG TCT CTG GAG GAA TTC     523
Ile Gly Leu Leu Asp Val Phe Thr Pro Ala Arg Ser Leu Glu Glu Phe
     85                  90                  95

AAT GAT GTG TAT CTG GTG ACC CAT CTC ATG GGG GCA GAT CTG AAC AAC     571
Asn Asp Val Tyr Leu Val Thr His Leu Met Gly Ala Asp Leu Asn Asn
100                 105                 110                 115

ATT GTG AAA TGT CAG AAG CTT ACA GAT GAC CAT GTT CAG TTC CTT ATC     619
Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln Phe Leu Ile
                120                 125                 130

TAC CAA ATT CTC CGA GGT CTA AAG TAT ATA CAT TCA GCT GAC ATA ATT     667
Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala Asp Ile Ile
            135                 140                 145

CAC AGG GAC CTA AAA CCT AGT AAT CTA GCT GTG AAT GAA GAC TGT GAG     715
His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu Asp Cys Glu
        150                 155                 160

CTG AAG ATT CTG GAT TTT GGA CTG GCT CGG CAC ACA GAT GAT GAA ATG     763
Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp Asp Glu Met
    165                 170                 175
```

```
ACA GGC TAC GTG GCC ACT AGG TGG TAC AGG GCT CCT GAG ATC ATG CTG     811
Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu Ile Met Leu
180                 185                 190                 195

AAC TGG ATG CAT TAC AAC CAG ACA GTT GAT ATT TGG TCA GTG GGA TGC     859
Asn Trp Met His Tyr Asn Gln Thr Val Asp Ile Trp Ser Val Gly Cys
            200                 205                 210

ATA ATG GCC GAG CTG TTG ACT GGA AGA ACA TTG TTT CCT GGT ACA GAC     907
Ile Met Ala Glu Leu Leu Thr Gly Arg Thr Leu Phe Pro Gly Thr Asp
        215                 220                 225

CAT ATT GAT CAG TTG AAG CTC ATT TTA AGA CTC GTT GGA ACC CCA GGG     955
His Ile Asp Gln Leu Lys Leu Ile Leu Arg Leu Val Gly Thr Pro Gly
    230                 235                 240

GCT GAG CTT TTG AAG AAA ATC TCC TCA GAG TCT GCA AGA AAC TAT ATT     1003
Ala Glu Leu Leu Lys Lys Ile Ser Ser Glu Ser Ala Arg Asn Tyr Ile
245                 250                 255

CAG TCT TTG ACT CAG ATG CCG AAG ATG AAC TTT GCG AAT GTA TTT ATT     1051
Gln Ser Leu Thr Gln Met Pro Lys Met Asn Phe Ala Asn Val Phe Ile
260                 265                 270                 275

GGT GCC AAT CCC CTG GCT GTC GAC TTG CTG GAG AAG ATG CTT GTA TTG     1099
Gly Ala Asn Pro Leu Ala Val Asp Leu Leu Glu Lys Met Leu Val Leu
            280                 285                 290

GAC TCA GAT AAG AGA ATT ACA GCG GCC CAA GCC CTT GCA CAT GCC TAC     1147
Asp Ser Asp Lys Arg Ile Thr Ala Ala Gln Ala Leu Ala His Ala Tyr
        295                 300                 305

TTT GCT CAG TAC CAC GAT CCT GAT GAT GAA CCA GTG GCC GAT CCT TAT     1195
Phe Ala Gln Tyr His Asp Pro Asp Asp Glu Pro Val Ala Asp Pro Tyr
    310                 315                 320

GAT CAG TCC TTT GAA AGC AGG GAC CTC CTT ATA GAT GAG TGG AAA AGC     1243
Asp Gln Ser Phe Glu Ser Arg Asp Leu Leu Ile Asp Glu Trp Lys Ser
325                 330                 335
```

```
CTG ACC TAT GAT GAA GTC ATC AGC TTT GTG CCA CCA CCC CTT GAC CAA     1291
Leu Thr Tyr Asp Glu Val Ile Ser Phe Val Pro Pro Pro Leu Asp Gln
340                 345                 350                 355

GAA GAG ATG GAG TCC TGAGCACCTG GTTTCTGTTC TGTTGATCCC ACTTCACTGT     1346
Glu Glu Met Glu Ser
                360

GAGGGGAAGG CCTTTTCACG GGAACTCTCC AAATATTATT CAAGTGCCAA AAAGGTCCAG   1406

CAATTTCGTT ACTTATG                                                  1423
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 360 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Met Ser Gln Glu Arg Pro Thr Phe Tyr Arg Gln Glu Leu Asn Lys Thr
 1               5                  10                  15

Ile Trp Glu Val Pro Glu Arg Tyr Gln Asn Leu Ser Pro Val Gly Ser
            20                  25                  30

Gly Ala Tyr Gly Ser Val Cys Ala Ala Phe Asp Thr Lys Thr Gly Leu
        35                  40                  45

Arg Val Ala Val Lys Lys Leu Ser Arg Pro Phe Gln Ser Ile Ile His
    50                  55                  60

Ala Lys Arg Thr Tyr Arg Glu Leu Arg Leu Leu Lys His Met Lys His
65                  70                  75                  80

Glu Asn Val Ile Gly Leu Leu Asp Val Phe Thr Pro Ala Arg Ser Leu
                85                  90                  95
```

```
Glu Glu Phe Asn Asp Val Tyr Leu Val Thr His Leu Met Gly Ala Asp
            100                 105                 110

Leu Asn Asn Ile Val Lys Cys Gln Lys Leu Thr Asp Asp His Val Gln
        115                 120                 125

Phe Leu Ile Tyr Gln Ile Leu Arg Gly Leu Lys Tyr Ile His Ser Ala
    130                 135                 140

Asp Ile Ile His Arg Asp Leu Lys Pro Ser Asn Leu Ala Val Asn Glu
145                 150                 155                 160

Asp Cys Glu Leu Lys Ile Leu Asp Phe Gly Leu Ala Arg His Thr Asp
                165                 170                 175

Asp Glu Met Thr Gly Tyr Val Ala Thr Arg Trp Tyr Arg Ala Pro Glu
            180                 185                 190

Ile Met Leu Asn Trp Met His Tyr Asn Gln Thr Val Asp Ile Trp Ser
        195                 200                 205

Val Gly Cys Ile Met Ala Glu Leu Leu Thr Gly Arg Thr Leu Phe Pro
    210                 215                 220

Gly Thr Asp His Ile Asp Gln Leu Lys Leu Ile Leu Arg Leu Val Gly
225                 230                 235                 240

Thr Pro Gly Ala Glu Leu Leu Lys Lys Ile Ser Ser Glu Ser Ala Arg
                245                 250                 255

Asn Tyr Ile Gln Ser Leu Thr Gln Met Pro Lys Met Asn Phe Ala Asn
            260                 265                 270

Val Phe Ile Gly Ala Asn Pro Leu Ala Val Asp Leu Leu Glu Lys Met
        275                 280                 285

Leu Val Leu Asp Ser Asp Lys Arg Ile Thr Ala Ala Gln Ala Leu Ala
    290                 295                 300
```

His Ala Tyr Ph Ala Gln Tyr His Asp Pro Asp Asp Glu Pro Val Ala
305 310 315 320

Asp Pro Tyr Asp Gln Ser Phe Glu Ser Arg Asp Leu Leu Ile Asp Glu
325 330 335

Trp Lys Ser Leu Thr Tyr Asp Glu Val Ile Ser Phe Val Pro Pro Pro
340 345 350

Leu Asp Gln Glu Glu Met Glu Ser
355 360

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGCCCTCGAG ATGTCTCAGG AGAGGCCCAC G 31

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCCAGTCCAA AATCCAGAAT C 21

WHAT IS CLAIMED IS

1. An isolated nucleic acid molecule encoding a cytokine suppressive anti-inflammatory drug binding protein.

2. The molecule according to Claim 1 wherein said nucleic acid is DNA.

3. The molecule according to Claim 2 having the sequence characterized by Figures 21 and 22.

4. An isolated protein which is a human CSBP.

5. The protein according to Claim 4 characterized by the amino acid sequence Figure 16.

6. The protein according to Claim 4 further characterized as being isolatable from human monocytes, having M_W of about 43,000 and having a pI of about 4.5.

7. A vector comprising the nucleic acid of Claim 1.

8. The vector according to Claim 7 which is a plasmid.

9. The plasmid according to Claim 8 which is a cloning plasmid.

10. The plasmid according to Claim 8 which is an expression plasmid.

11. A recombinant host cell comprising the vector of Claim 7.

12. The host cell according to Claim 11 which is a prokaryotic cell.

13. The host cell according to Claim 11 which is an eukayrotic cell.

14. A method for the production of CSBP comprising culturing a recombinant host cell capable of expressing CSBP in a medium and under conditions sufficient form such expression and recovering CSBP from the host cell.

15. A method for identifying a compound as a CSAID comprising: (a) contacting a known CSAID labelled with an analytically detectable reagent with a CSBP under conditions sufficient to form a CSAID/CSBP complex; (b) contacting said complex with a sample comprising a compound to be identified; and (c) identifying the compound as a CSAID by detecting the ability of said compound to alter the amount of labelled CSAID in said complex.

16. The method according to Claim 15 wherein the CSBP is in a form selected form the group consisting of whole cells, cytosolic cell fractions, membrane cell fractions, and purified or partially purified form.

17. A method for identifying a compound as a CSAID comprising:

a. forming soluble cytosolic fraction from a cell expressing a CSBP.

b. contact said fraction with a CSAID labelled with an analytically detectable reagent under conditions sufficient to form a reagent CSAID/CSBP complex;

c. contacting said complex with a sample containing a CSAID; and

d. detecting the CSAID by measuring a decrease of the amount of reagent in the labelled CSAID/CSBP complex.

18. The method according to Claim 17 wherein said cell is a human monocyte.

19. The method according to Claim 17 wherein said cell is a recombinant host cell.

20. The method according to Claim 17 wherein said reagent is a radioactive label.

21. A method for identifying ligands capable of binding to a CSBP comprising: contacting a recombinant host cell expressing a CSBP with a ligand to be identified under conditions to permit binding and detecting the presence of any ligand-bound protein.

22. The method according to Claim 21 wherein the recombinant host cell expresses said CSBP at its cell surface.

23. The method according to Claim 21 wherein the protein or a membrane fraction containing the protein is isolated from said cell prior to contacting with the ligand to be identified.

24. An antagonist or agonist compound identified by the method of Claim 15.

25. A pharmaceutical composition comprising a compound identified by the method of Claim 15 and a pharmaceutically acceptable carrier.

26. A antisense oligonucleotide having sequence capable of binding specifically with any sequence of an mRNA molecule which encodes the human CSBP containing the amino acid sequence of Figure 16 so as to prevent the translation thereof.

27 An antibody directed to the human CSBP of Claim 5.

28. The antibody according to Claim 26 which is a monoclonal antibody.

29. A transgenic non-human mammal capable of expressing in any cell thereof the DNA of Claim 3.

30. A method of screening compounds to identify those compounds which bind to a human CSBP comprising contacting the fusion protein comprising a CSBP domain and a binding protein/ligand binding indicator domain with a pluarality f compound, under conditions to permit binding to the CSBP domain, and identifyting those candidate drugs capable of enhancing or inhibiting the activity of the protein/ligand binding indicator domain.

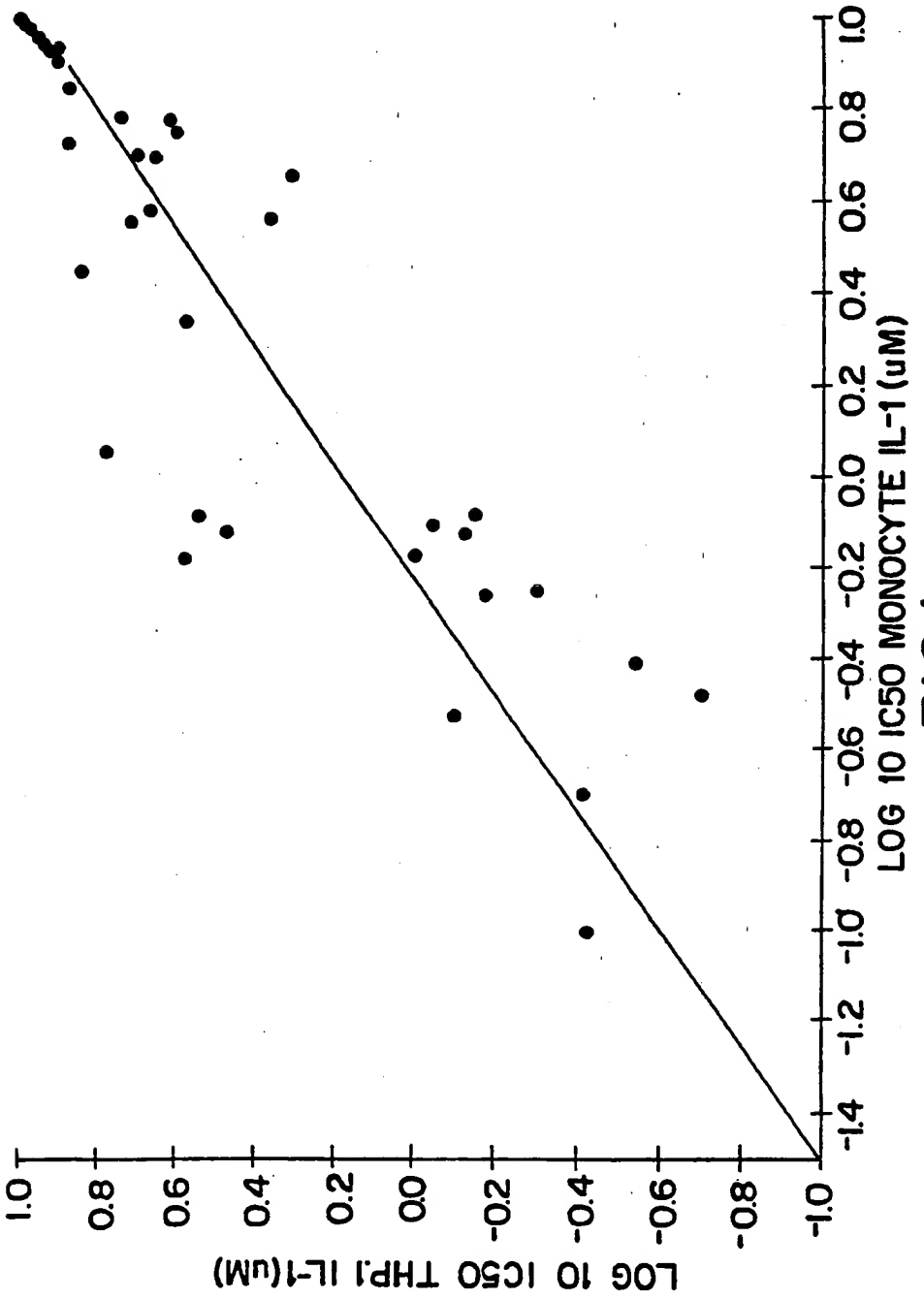
LOG 10 IC50 THP.1 IL-1(uM)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
LOG 10 IC50 MONOCYTE IL-1 (uM)
-1.4
-1.2
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0

FIG. 1

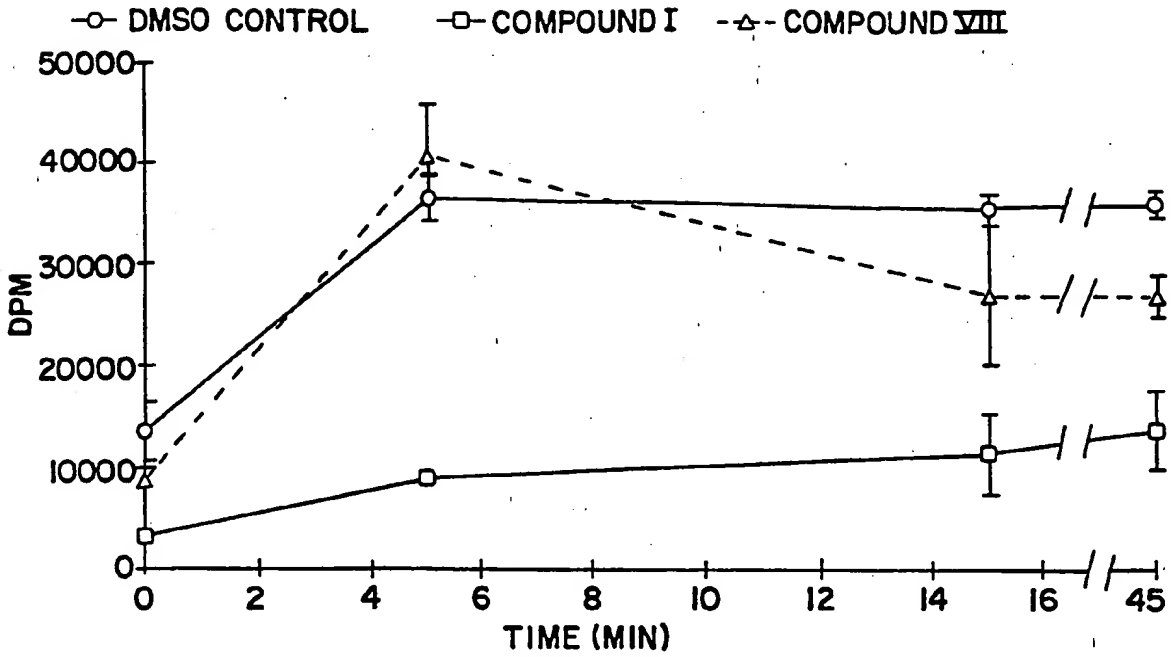
DMSO CONTROL
COMPOUND I
COMPOUND VIII
DPM
50000
40000
30000
20000
10000
0
0
2
4
6
8
10
12
14
16
45
TIME (MIN)

FIG.2

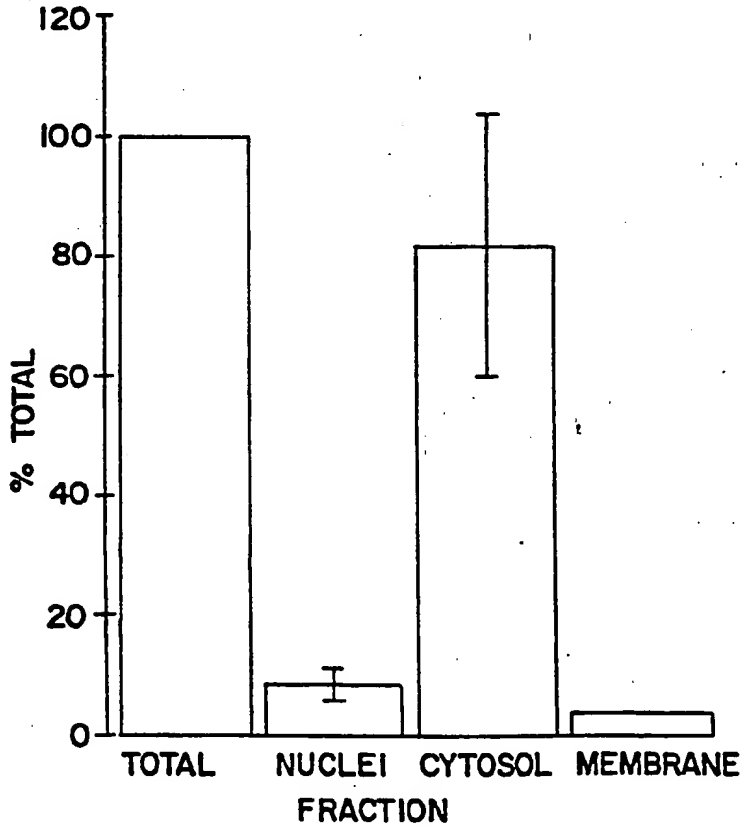
% TOTAL
120
100
80
60
40
20
0
TOTAL
NUCLEI
CYTOSOL
MEMBRANE
FRACTION

FIG.3

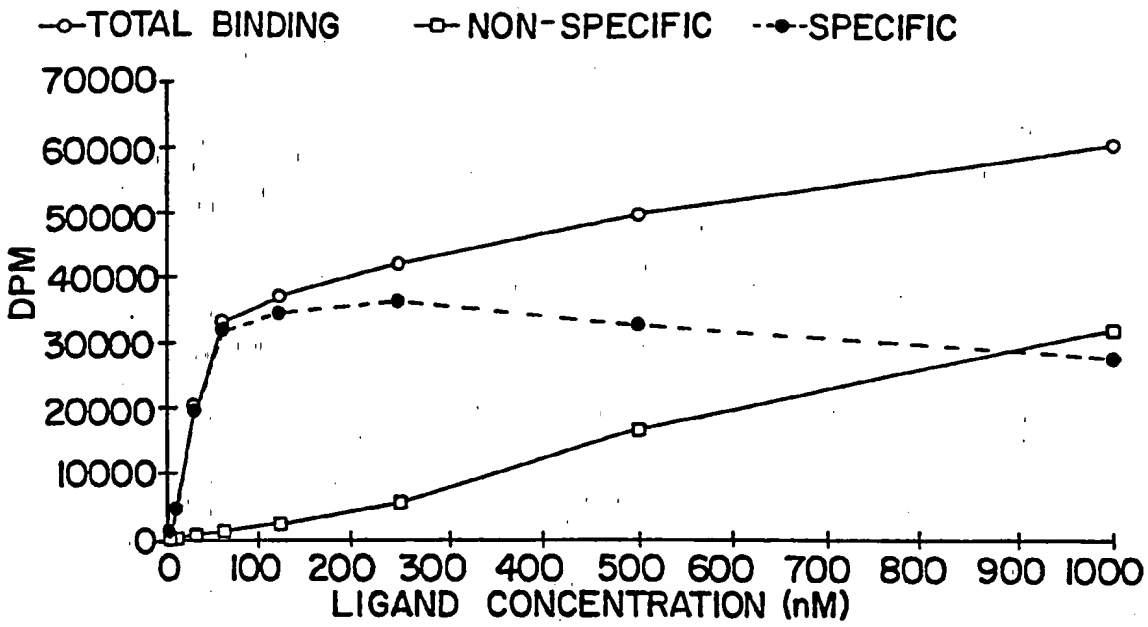
TOTAL BINDING
NON-SPECIFIC
SPECIFIC
DPM
70000
60000
50000
40000
30000
20000
10000
0
0 100 200 300 400 500 600 700 800 900 1000
LIGAND CONCENTRATION (nM)

FIG.4A

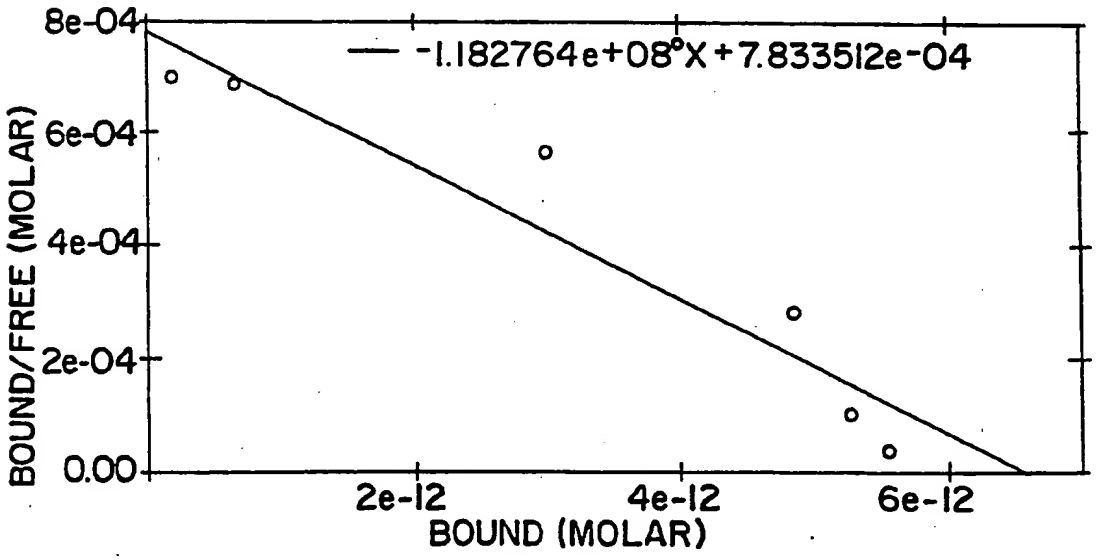
-1.182764e+08*X+7.833512e-04
BOUND/FREE (MOLAR)
8e-04
6e-04
4e-04
2e-04
0.00
2e-12
4e-12
6e-12
BOUND (MOLAR)

FIG.4B

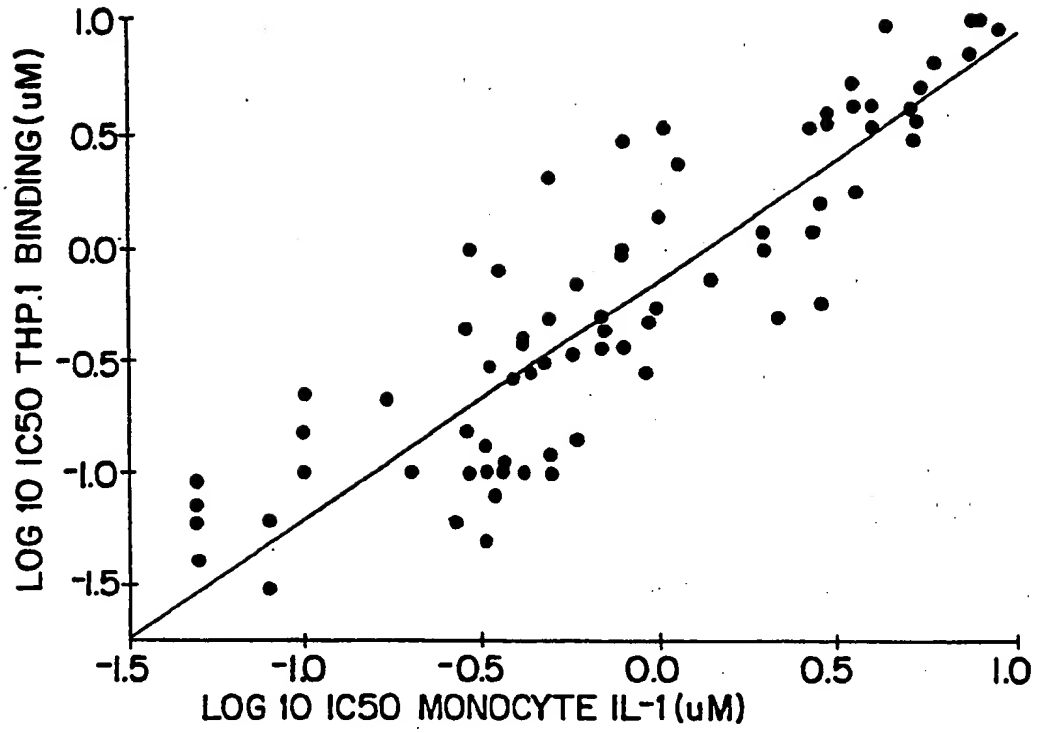
LOG 10 IC50 THP.1 BINDING (uM)
1.0
0.5
0.0
-0.5
-1.0
-1.5
-1.5
-1.0
-0.5
0.0
0.5
1.0
LOG 10 IC50 MONOCYTE IL-1 (uM)

FIG.5

XI

VALUES ARE IC_{50},μM

COMPOUND	R1/R2	X-Y	IL-1	TNF	THP-1 CYTOSOL BINDING
XI(a)	4-pyridyl/4-FPh	$-CH_2CH_2S-$	0.5	0.4	<0.1
XI(b)	4-FPh/4-pyridyl	$-CH_2CH_2S-$	>5	>5	10
XI(c)	4-pyridyl/4-FPh	$-CH_2CH_2CH_2-$	0.2	0.2	<0.1
XI(d)	4-FPh/4-pyridyl	$-CH_2CH_2CH_2-$	>5	>5	>10
XI(e)	4-pyridyl/4-MeSPh	$-CH_2CH_2CH_2-$	2.7	2.7	3.4
XI(f)	4-MeSPh/4-pyridyl	$-CH_2CH_2CH_2-$	>5	>5	5
XI(g)	4-pyridyl/4-FPh	$-CH_2CH_2OAC,H$	0.5	3	0.5
XI(h)	4-FPh/4-pyridyl	$-CH_2CH_2OAC,H$	5	5	>10

FIG.6

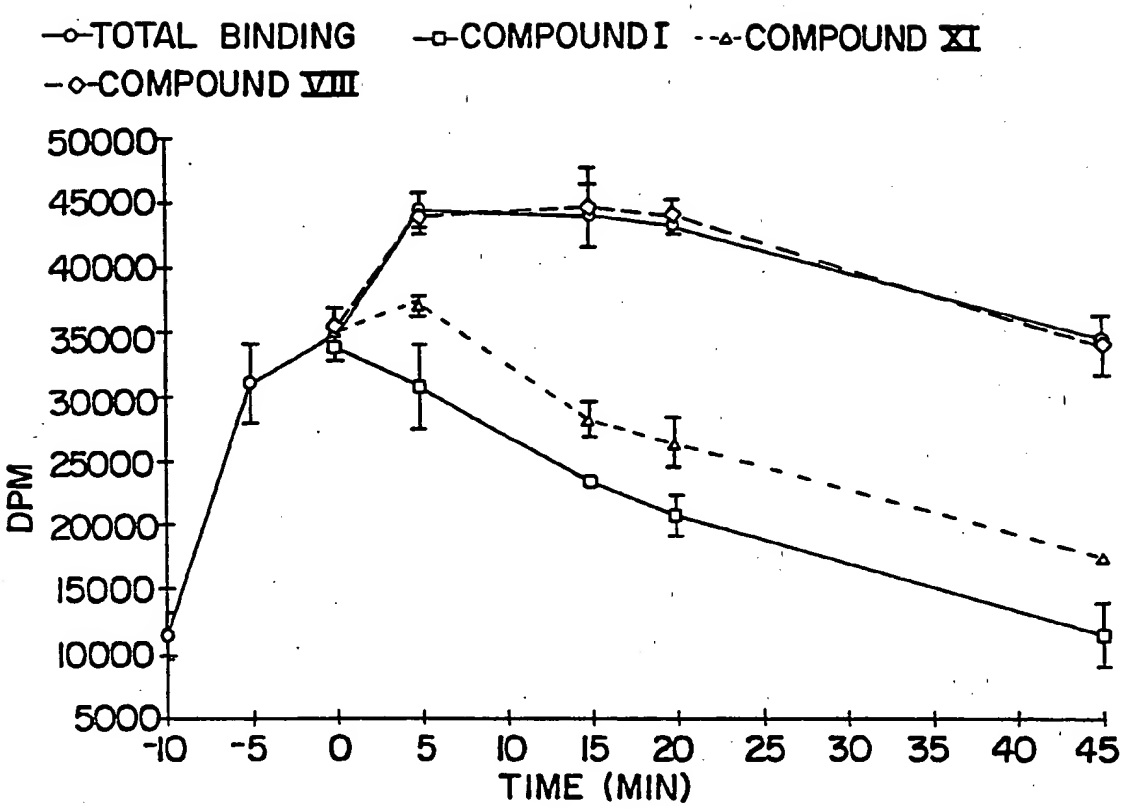
TOTAL BINDING
COMPOUND I
COMPOUND XI
COMPOUND VIII
DPM
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
-10
-5
0
5
10
15
20
25
30
35
40
45
TIME (MIN)

FIG.7

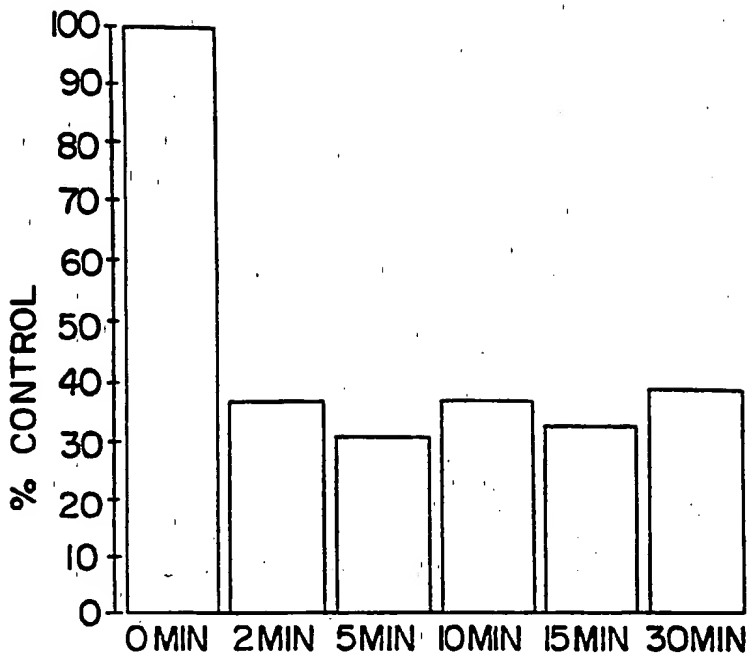
100
90
80
70
60
50
40
30
20
10
0
% CONTROL
0 MIN
2 MIN
5 MIN
10 MIN
15 MIN
30 MIN

FIG.8A

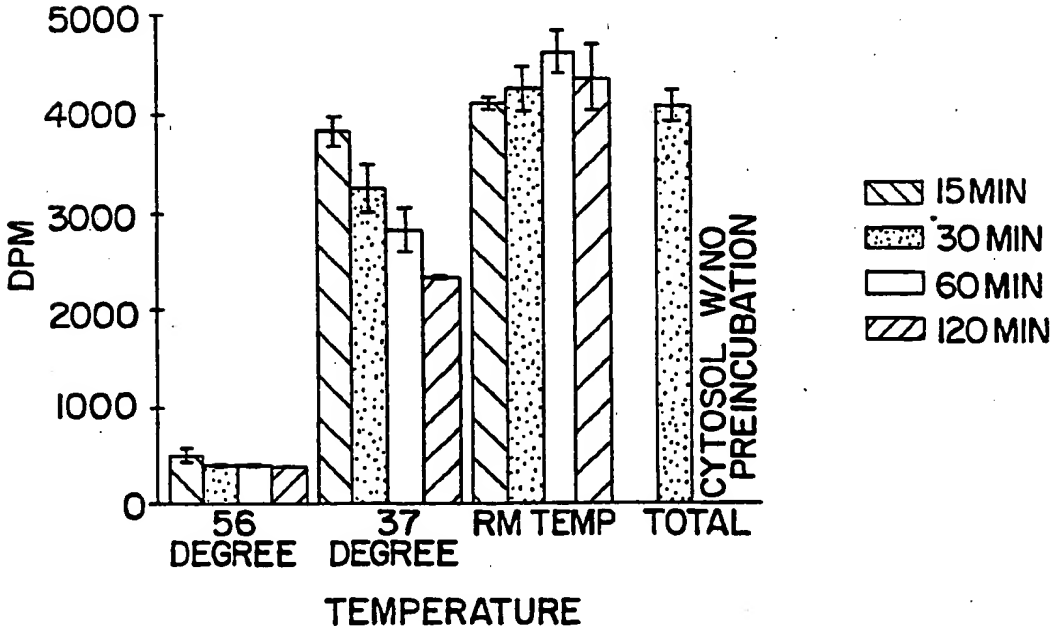
5000
4000
3000
2000
1000
0
DPM
56 DEGREE
37 DEGREE
RM TEMP
TOTAL
TEMPERATURE
CYTOSOL W/NO PREINCUBATION
15 MIN
30 MIN
60 MIN
120 MIN

FIG.8B

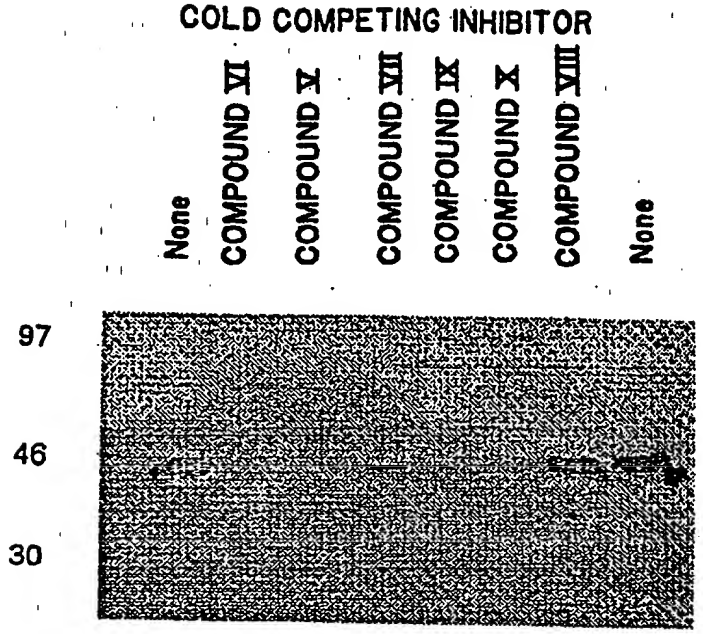
COLD COMPETING INHIBITOR
None
COMPOUND VI
COMPOUND V
COMPOUND III
COMPOUND IX
COMPOUND X
COMPOUND VIII
None
97
46
30

FIG.9

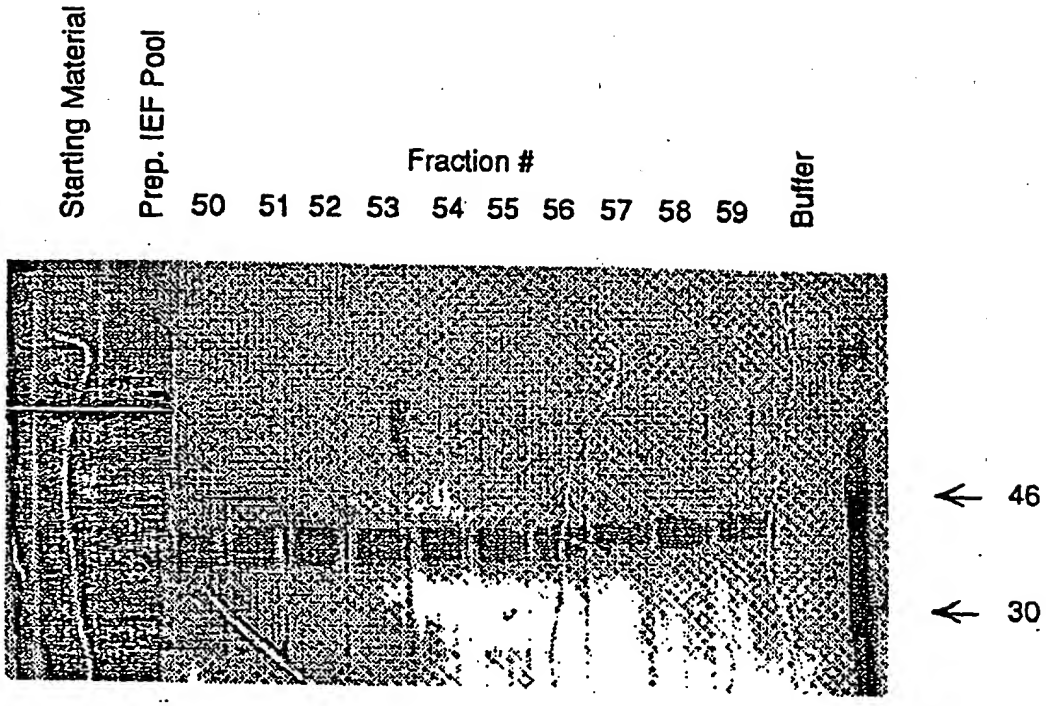
Starting Material
Prep. IEF Pool
Fraction #
50 51 52 53 54 55 56 57 58 59
Buffer
46
30

FIG.11A

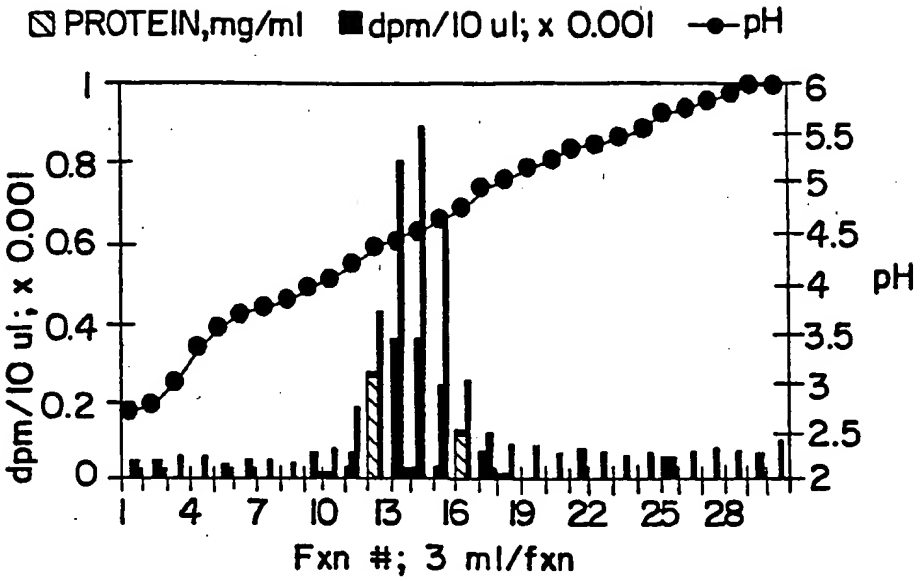

FIG. 10

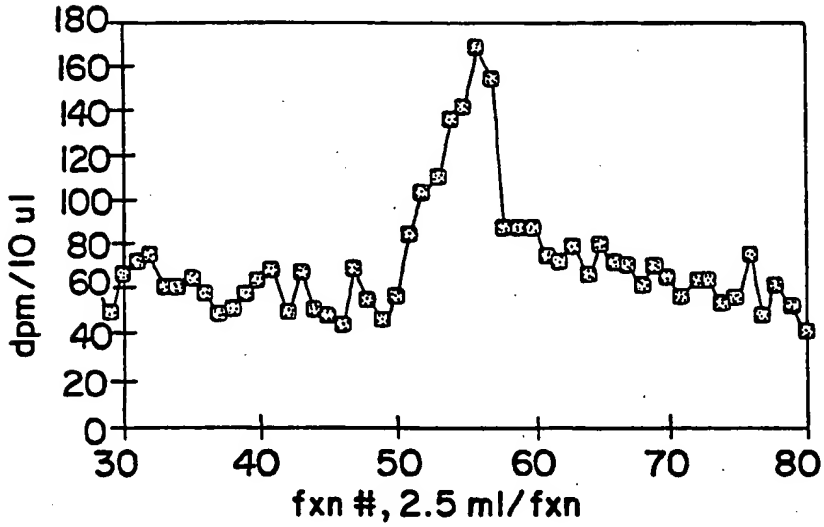

FIG. 11B

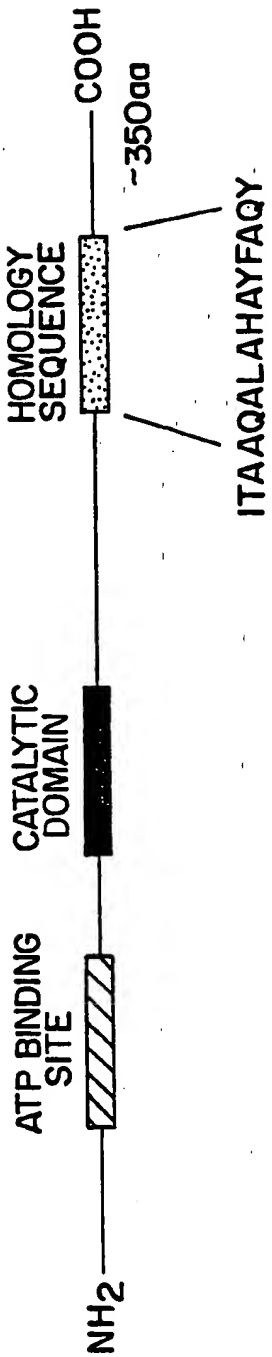
COOH
~350aa
HOMOLOGY SEQUENCE
ITAAQALAHAYFAQY
CATALYTIC DOMAIN
ATP BINDING SITE
NH2

FIG.12

```
1    AACATTGTGAAATGTCAGAAGCTTACAGATGACCATGTTCAGTTCCTTATCTACCAAATT   60
     AsnIleValLysCysGlnLysLeuThrAspAspHisValGlnPheLeuIleTyrGlnIle

61   CTCCGAGGTCTAAAGTATATACATTCAGCTGACATAATTCACAGGGACCTAAAACCTAGT   120
     LeuArgGlyLeuLysTyrIleHisSerAlaAspIleIleHisArgAspLeuLysProSer

121  AATCTAGCTGTGAATGAAGACTGTGAGCTGAAGATTCTGGATTTTGGACTGGCTCGGCAC   180
     AsnLeuAlaValAsnGluAspCysGluLeuLysIleLeuAspPheGlyLeuAlaArgHis

181  ACAGATGATGAAATGACAGGCTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATCATG   240
     ThrAspAspGluMetThrGlyTyrValAlaThrArgTrpTyrArgAlaProGluIleMet

241  CTGAACTGGATGCATTACAACCAGACAGGTGGTATTTGGGTCAAG  285
     LeuAsnTrpMetHisTyrAsnGlnThrGlyGlyIleTrpValLys
```

FIG. 13

CAAGTCCCAATCCTCCCCAACCACAGCAAGTTGAATTTATCAACCATGTTGGGTTGTAAA

TGCTCGTGTGATTTCCTACAAGAAATACCTGCTCTGAATATTTTTGTAATAAAGGTCTTT

GCACATGTGACCCACAATACGTGTTAGGAGCCTGCATGCTCTGGAAGCCTGGACTCTAAG

CTGGAGCTCTTGGAAGAGCTCTTCGGTTTCTGAGCATAATGCTCCCATCTCCTGATTTCT

CTGAACAGAAAACAAAAGAGAGAATGAGGGAAATTGCTATTTTATTTGTATTCATGAACT

TGGCTGTAATCAGTTATGCCGTATAGGATGTCAGACAATACCACTGGTTAAAATAAAGCC

TATTTTTCAAATTTAAAAAAAAAAAAAAAAA

FIG.14

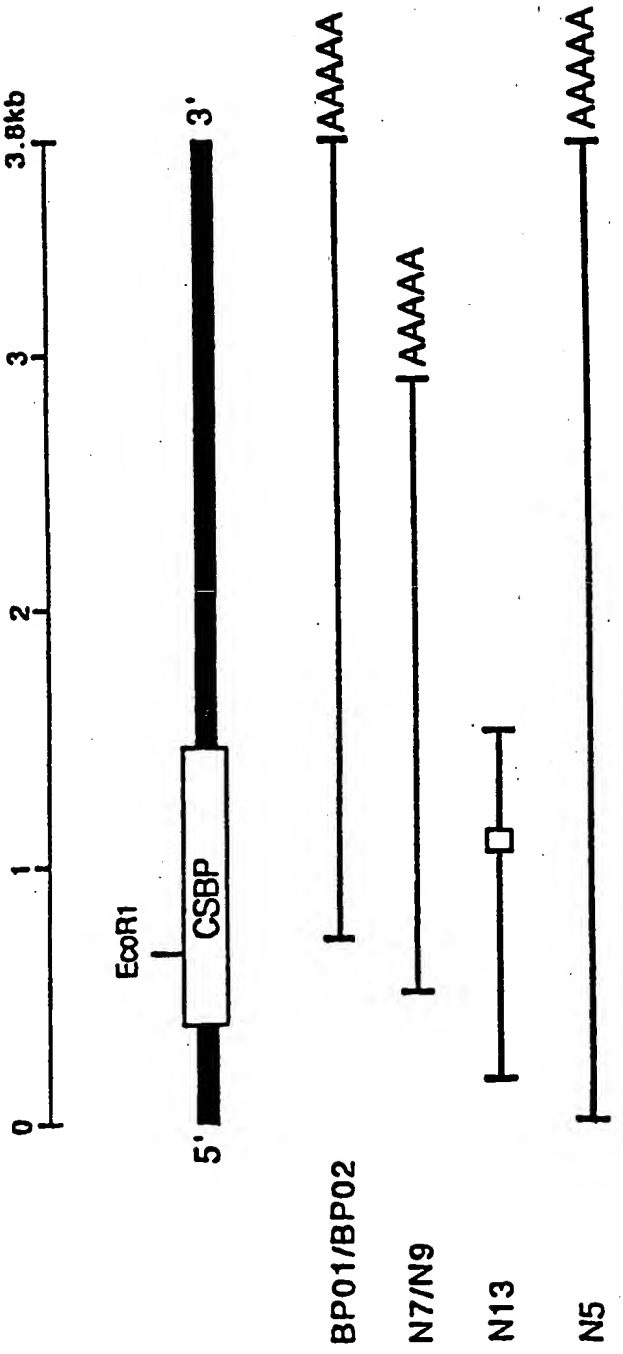
3.8kb
0
1
2
3
5'
3'
EcoR1
CSBP
AAAAA
AAAAA
AAAAA
BP01/BP02
N7/N9
N13
N5

FIG.15

```
..355nts..GCCGCTGGAAAATGTCTCAGGAGAGGCCCACGTTCTACCGGCAGGAGCTGAACAAGACAATCTGG  420
                    M  S  Q  E  R  P  T  F  Y  R  Q  E  L  N  K  T  I  W   18

GAGGTGCCCGAGCGTTACCAGAACCTGTCTCCAGTGGGCTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGAC  495
E  V  P  E  R  Y  Q  N  L  S  P  V  G  S  G  A  Y  G  S  V  C  A  A  F  D   43

ACAAAAACGGGGTTACGTGTGGCAGTGAAGAAGCTCTCCAGACCATTTCAGTCCATCATTCATGCGAAAAGAACC  570
T  K  T  G  L  R  V  A  V  K  K  L  S  R  P  F  Q  S  I  I  H  A  K  R  T   68

TACAGAGAACTGCGGTTACTTAAACATATGAAACATGAAAATGTGATTGGTCTGTTGGACGTTTTTACACCTGCA  645
Y  R  E  L  R  L  L  K  H  M  K  H  E  N  V  I  G  L  L  D  V  F  T  P  A   93

AGGTCTCTGGAGGAATTCAATGATGTGTATCTGGTGACCCATCTCATGGGGGCAGATCTGAACAACATTGTGAAA  720
R  S  L  E  E  F  N  D  V  Y  L  V  T  H  L  M  G  A  D  L  N  N  I  V  K  118

TGTCAGAAGCTTACAGATGACCATGTTCAGTTCCTTATCTACCAAATTCTCCGAGGTCTAAAGTATATACATTCA  795
C  Q  K  L  T  D  D  H  V  Q  F  L  I  Y  Q  I  L  R  G  L  K  Y  I  H  S  143

GCTGACATAATTCACAGGGACCTAAAACCTAGTAATCTAGCTGTGAATGAAGACTGTGAGCTGAAGATTCTGGAT  870
A  D  I  I  H  R  D  L  K  P  S  N  L  A  V  N  E  D  C  E  L  K  I  L  D  168

TTTGGACTGGCTCGGCACACAGATGATGAAATGACAGGCTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATC  945
F  G  L  A  R  H  T  D  D  E  M  T  G  Y  V  A  T  R  W  Y  R  A  P  E  I  193
```

FIG.16A

```
ATGCTGAACTGGATGCATTACAACCAGACAGTTGATATTTGGTCAGTGGGATGCATAATGGCCGAGCTGTTGACT  1020
M  L  N  W  M  H  Y  N  Q  T  V  D  I  W  S  V  G  C  I  M  A  E  L  L  T      218

GGAAGAACATTGTTTCCTGGTACAGACCATATTAACCAGCTTCAGCAGATTATGCGTCTGACAGGAACACCCCCC  1095
G  R  T  L  F  P  G  T  D  H  I  N  Q  L  Q  Q  I  M  R  L  T  G  T  P  P      243

GCTTATCTCATTAACAGGATGCCAAGCCATGAGGCAAGAAACTATATTCAGTCTTTGACTCAGATGCCGAAGATG  1170
A  Y  L  I  N  R  M  P  S  H  E  A  R  N  Y  I  Q  S  L  T  Q  M  P  K  M      268

AACTTTGCGAATGTATTTATTGGTGCCAATCCCCTGGCTGTCGACTTGGTGGAGAAGATGCTTGTATTGGACTCA  1245
N  F  A  N  V  F  I  G  A  N  P  L  A  V  D  L  L  E  K  M  L  V  L  D  S      293

GATAAGAGAATTACAGCGGCCCAAGCCCTTGCACATGCCTACTTTGCTCAGTACCACGATCCTGATGAACCA  1320
D  K  R  I  T  A  Q  A  L  A  H  A  Y  F  A  Q  Y  H  D  P  D  D  E  P      318

GTGGCCGATCCTTATGATCAGTCCTTTGAAAGCAGGGACCTCCTTATAGATGAGTGGAAAAGCCTGACCTATGAT  1395
V  A  D  P  Y  D  Q  S  F  E  S  R  D  L  L  I  D  E  W  K  S  L  T  Y  D      343

GAAGTCATCAGCTTTGTGCCACCACCCCTTGACCAAGAAGATGGAGTCCTGAGCACCT...2330nts..
E  V  I  S  F  V  P  P  P  L  D  Q  E  E  M  E  S  *
```

FIG.16B

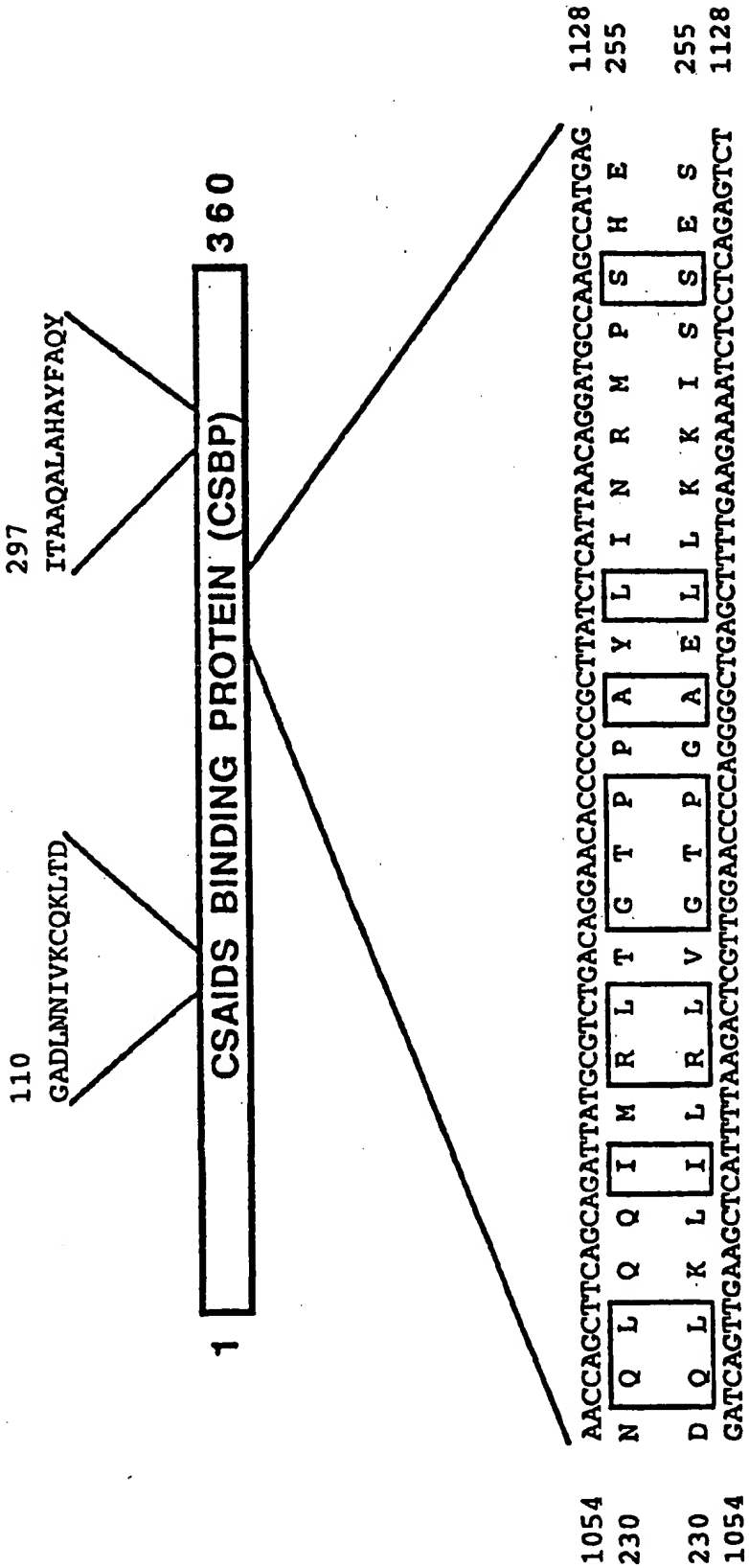
110
GADLNNIVKCQKLTD
297
ITAAQALAHAYFAQY
1
CSAIDS BINDING PROTEIN (CSBP)
360
1054 AACCAGCTTCAGCAGATTATGCGTCTGACAGGAACACCCCCGCTTATCTCATTAACAGGATGCCAAGCCATGAG 1128
230 N Q L Q Q I M R L T G T P P A Y L I N R M P S H E 255
230 D Q L K L I L R L V G T P G A E L L K K I S S E S 255
1054 GATCAGTTGAAGCTCATTTTAAGACTCGTTGGAACCCCAGGGGCTGAGCTTTGAAGAAATCTCCTCAGAGTCT 1128

FIG.17

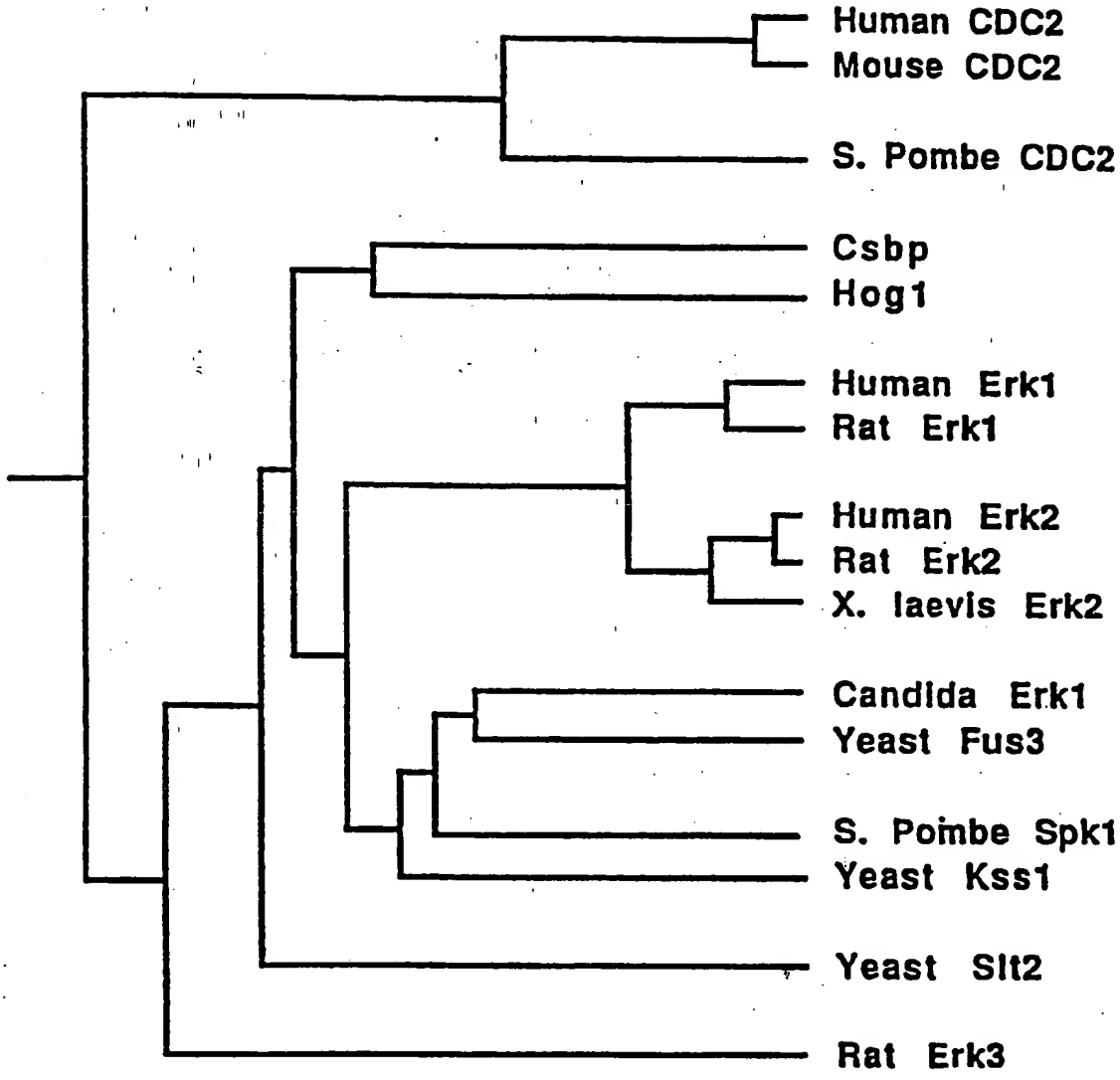
Human CDC2
Mouse CDC2
S. Pombe CDC2
Csbp
Hog1
Human Erk1
Rat Erk1
Human Erk2
Rat Erk2
X. laevis Erk2
Candida Erk1
Yeast Fus3
S. Pombe Spk1
Yeast Kss1
Yeast Slt2
Rat Erk3

FIG.18

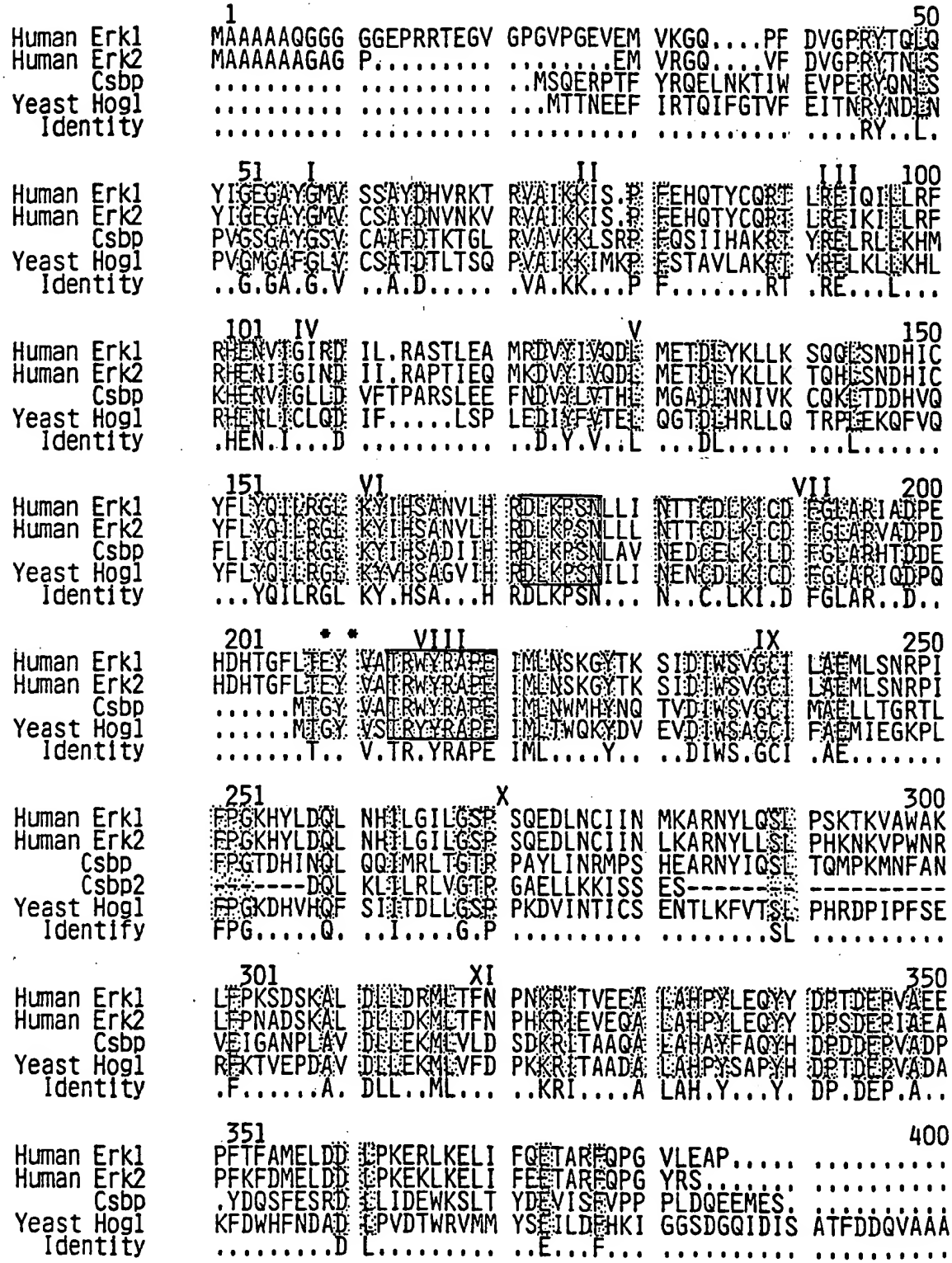
1 50
Human Erk1 MAAAAAQGGG GGEPRRTEGV GPGVPGEVEM VKGQ....PF DVGPRYTQLQ
Human Erk2 MAAAAAAGAG P..... .....EM VRGQ....VF DVGPRYTNLS
Csbp ....MSQERPTF YRQELNKTIW EVPERYQNLS
Yeast Hog1 ....MTTNEEF IRTQIFGTVF EITNRYNDLN
Identity ....RY..L.
51 I II III 100
Human Erk1 YIGEGAYGMV SSAYDHVRKT RVAIKKIS.P FEHQTYCQRT LREIQILLRF
Human Erk2 YIGEGAYGMV CSAYDNVNKV RVAIKKIS.P FEHQTYCQRT LREIKILLRF
Csbp PVGSGAYGSV CAAFDTKTGL RVAVKKLSRP FQSIIHAKRT YRELRLLKHM
Yeast Hog1 PVGMGAFGLV CSATDTLTSQ PVAIKKIMKP FSTAVLAKRT YRELKLLKHL
Identity ..G.GA.G.V ..A.D..... .VA.KK...P F.....RT .RE...L...
101 IV V 150
Human Erk1 RHENVIGIRD IL.RASTLEA MRDVYIVQDL METDLYKLLK SQQLSNDHIC
Human Erk2 RHENIIGIND II.RAPTIEQ MKDVYIVQDL METDLYKLLK TQHLSNDHIC
Csbp KHENVIGLLD VFTPARSLEE FNDVYLVTHL MGADLNNIVK CQKLTDDHVQ
Yeast Hog1 RHENLICLQD IF.....LSP LEDIYFVTEL QGTDLHRLLQ TRPLEKQFVQ
Identity .HEN.I...D ....D.Y.V..L ...DL..... ...L.....
151 VI VII 200
Human Erk1 YFLYQILRGL KYIHSANVLH RDLKPSNLLI NTTCDLKICD FGLARIADPE
Human Erk2 YFLYQILRGL KYIHSANVLH RDLKPSNLLL NTTCDLKICD FGLARVADPD
Csbp FLIYQILRGL KYIHSADIIH RDLKPSNLAV NEDCELKILD FGLARHTDDE
Yeast Hog1 YFLYQILRGL KYVHSAGVIH RDLKPSNILI NENCDLKICD FGLARIQDPQ
Identity ...YQILRGL KY.HSA...H RDLKPSN... N..C.LKI.D FGLAR..D..
201 * * VIII IX 250
Human Erk1 HDHTGFLTEY VATRWYRAPE IMLNSKGYTK SIDIWSVGCI LAEMLSNRPI
Human Erk2 HDHTGFLTEY VATRWYRAPE IMLNSKGYTK SIDIWSVGCI LAEMLSNRPI
Csbp .....MIGY VATRWYRAPE IMLNWMHYNQ TVDIWSVGCI MAELLTGRTL
Yeast Hog1 .....MIGY VSTRYYRAPE IMLTWQKYDV EVDIWSAGCI FAEMIEGKPL
Identity .....T.. V.TR.YRAPE IML....Y.. ..DIWS.GCI .AE.....
251 X 300
Human Erk1 FPGKHYLDQL NHILGILGSP SQEDLNCIIN MKARNYLQSL PSKTKVAWAK
Human Erk2 FPGKHYLDQL NHILGILGSP SQEDLNCIIN LKARNYLLSL PHKNKVPWNR
Csbp FPGTDHINQL QQIMRLTGTP PAYLINRMPS HEARNYIQSL TQMPKMNFAN
Csbp2 -------DQL KLILRLVGTP GAELLKKISS ES---------- ----------
Yeast Hog1 FPGKDHVHQF SIITDLLGSP PKDVINTICS ENTLKFVTSL PHRDPIPFSE
Identify FPG.....Q. ..I....G.P ....SL .....
301 XI 350
Human Erk1 LFPKSDSKAL DLLDRMLTFN PNKRITVEEA LAHPYLEQYY DPTDEPVAEE
Human Erk2 LFPNADSKAL DLLDKMLTFN PHKRIEVEQA LAHPYLEQYY DPSDEPIAEA
Csbp VEIGANPLAV DLLEKMLVLD SDKRITAAQA LAHAYFAQYH DPDDEPVADP
Yeast Hog1 RFKTVEPDAV DLLEKMLVFD PKKRITAADA LAHPYSAPYH DPTDEPVADA
Identity .F.....A. DLL..ML... ..KRI....A LAH.Y...Y. DP.DEP.A..
351 400
Human Erk1 PFTFAMELDD LPKERLKELI FQETARFQPG VLEAP..... .....
Human Erk2 PFKFDMELDD LPKEKLKELI FEETARFQPG YRS..... .....
Csbp .YDQSFESRD LIDEWKSLT YDEVISFVPP PLDQEEMES. .....
Yeast Hog1 KFDWHFNDAD LPVDTWRVMM YSEILDFHKI GGSDGQIDIS ATFDDQVAAA
Identity .....D L..... ..E...F... ....

FIG.19

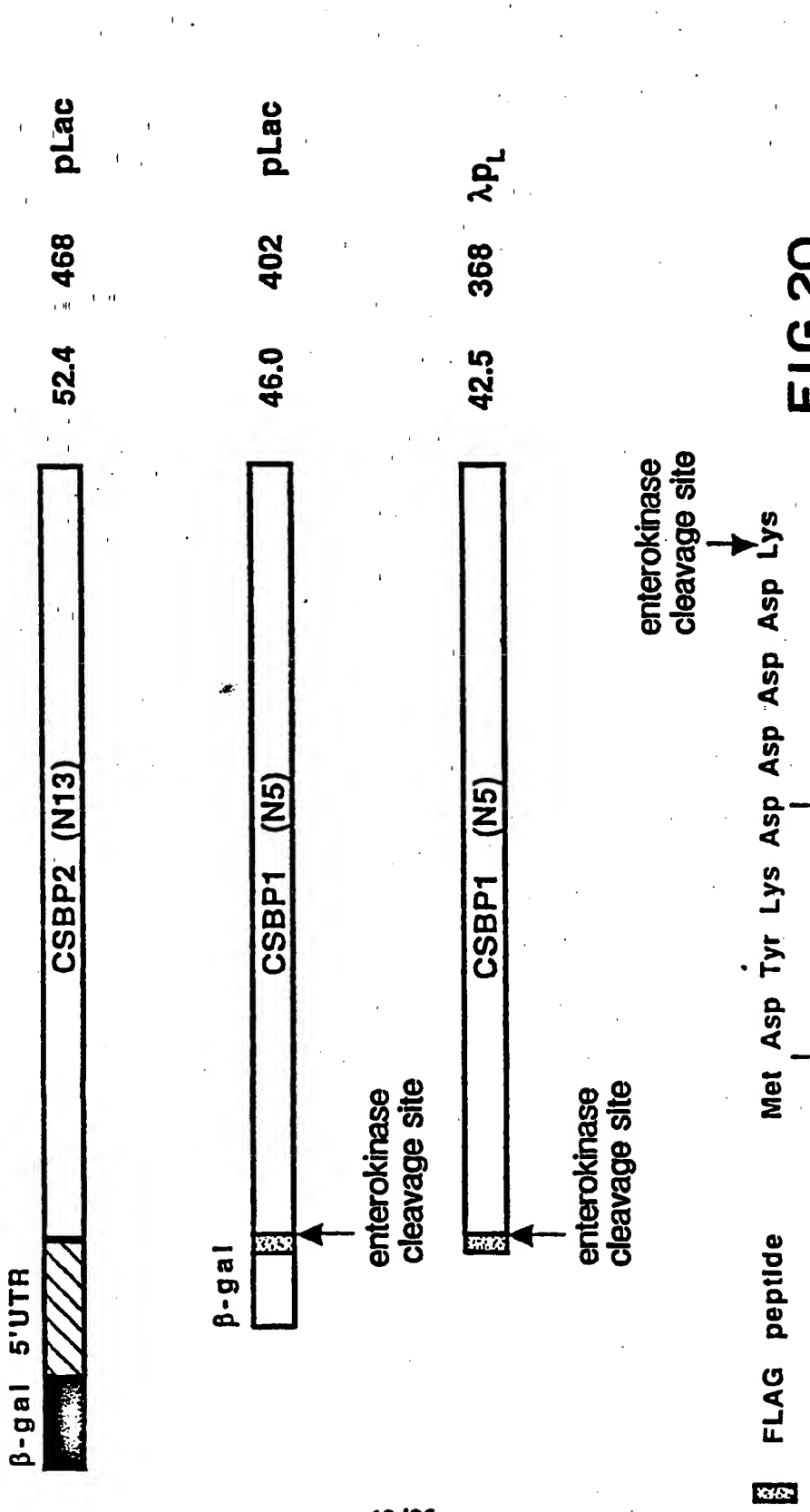
MW
AA
Prom ter
52.4
468
pLac
46.0
402
pLac
42.5
368
λp_L
β-gal 5'UTR
CSBP2 (N13)
β-gal
CSBP1 (N5)
enterokinase cleavage site
CSBP1 (N5)
enterokinase cleavage site
enterokinase cleavage site
FLAG peptide
Met Asp Tyr Lys Asp Asp Asp Asp Lys
M1 antibody binding site

FIG.20

```
1    GGAACCGCGACCACTGGAGCCTTAGCGGGCGCAGCAGCTGGAACGGGAGTACTGCGACGCAGCCCGGAGTCGGCC

76   TTGTAGGGGCGAAGGTGCAGGGAGATCGCGGCGGGCGCAGTCTTGAGCGCCGGAGCGCGTCCCTGCCCTTAGCGG

151  GGCTTGCCCCAGTCGCAGGGGCACATCCAGCCGCTGCGGCTGACAGCAGCCGCGCGCGCGGGAGTCTGCGGGGTC

226  GCGGCAGCCGCACCTGCGCGGGCGACCAGCGCAAGGTCCCCGCCCGGCTGGGCGGGCAGCAAGGGCCGGGGAGAG

301  GGTGCGGGTGCAGGCGGGGGCcCCACAGGGCCACCTTCTTGCCCGGCGGCTGCCGCTGGAAAATGTCTCAGGAGA
-19                                                                  MetSerGlnGluA

376  GGcCCACGTTCTACCGGCAGGAGCTGAACAAGACAATCTGGGAGGTGCCCGAGCGTTACCAGAACCTGTCTCCAG
 6   rgProThrPheTyrArgGlnGluLeuAsnLysThrIleTrpGluValProGluArgTyrGlnAsnLeuSerProV

451  TGGGCTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGACACAAAAACGGGGTTACGTGTGGCAGTGAAGAAGC
 31  alGlySerGlyAlaTyrGlySerValCysAlaAlaPheAspThrLysThrGlyLeuArgValAlaValLysLysL

526  TCTCCAGACCATTTCAGTCCATCATTCATGCGAAAAGAACCTACAGAGAACTGCGGTTACTTAAACATATGAAAC
 56  euSerArgProPheGlnSerIleIleHisAlaLysArgThrTyrArgGluLeuArgLeuLeuLysHisMetLysH
```

FIG.21A

```
601  ATGAAAATGTGATTGGTCTGTTGGACGTTTTTACACCTGCAAGGTCTCTGGAGGAATTCAATGATGTGTATCTGG
 81  isGluAsnValIleGlyLeuLeuAspValPheThrProAlaArgSerLeuGluGluPheAsnAspValTyrLeuV

676  TGACCCATCTCATGGGGGCAGATCTGAACAACATTGTGAAATGTCAGAAGCTTACAGATGACCATGTTCAGTTCC
106  alThrHisLeuMetGlyAlaAspLeuAsnAsnIleValLysCysGlnLysLeuThrAspAspHisValGlnPheL

751  TTATCTACCAAATTCTCCGAGGTCTAAAGTATATACATTCAGCTGACATAATTCACAGGGACCTAAAACCTAGTA
131  euIleTyrGlnIleLeuArgGlyLeuLysTyrIleHisSerAlaAspIleIleHisArgAspLeuLysProSerA

826  ATCTAGCTGTGAATGAAGACTGTGAGCTGAAGATTCTGGATTTTGGACTGGCTCGGCACACAGATGATGAAATGA
156  snLeuAlaValAsnGluAspCysGluLeuLysIleLeuAspPheGlyLeuAlaArgHisThrAspAspGluMetT

901  CAGGCTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATCATGCTGAACTGGATGCATTACAACCAGACAGTTG
181  hrGlyTyrValAlaThrArgTrpTyrArgAlaProGluIleMetLeuAsnTrpMetHisTyrAsnGlnThrValA

976  ATATTTGGTCAGTGGGATGCATAATGGCCGAGCTGTTGACTGGAAGAACATTGTTTCCTGGTACAGACCATATTA
206  spIleTrpSerValGlyCysIleMetAlaGluLeuLeuThrGlyArgThrLeuProGlyThrAspHisIleA

1051  AccagcttcagcagATTAtgcgtctgacaggaacACCCCccgcttatctcattaacaggatgccaagccatgagG
231   snGlnLeuGlnGlnIleMetArgLeuThrGlyThrProProAlaTyrLeuIleAsnArgMetProSerHisGluA

1126  CAAGAAACTATATTCAGTCTTTGACTCAGATGCCGAAGATGAACTTTGCGAATGTATTTATTGGTGCCAATCCCC
256   laArgAsnTyrIleGlnSerLeuThrGlnMetProLysMetAsnPheAlaAsnValPheIleGlyAlaAsnProL
```

FIG. 21B

```
1201  TGGCTGTCGACTTGCTGGAGAAGATGCTTGTATTGGACTCAGATAAGAGAATTACAGCGGCCCAAGCCCTTGCAC
 281  euAlaValAspLeuLeuGluLysMetLeuValLeuAspSerAspLysArgIleThrAlaAlaGlnAlaLeuAlaH

1276  ATGCCTACTTTGCTCAGTACCACGATCCTGATGATGAACCAGTGGCCGATCCTTATGATCAGTCCTTTGAAAGCA
 306  isAlaTyrPheAlaGlnTyrHisAspProAspAspGluProValAlaAspProTyrAspGlnSerPheGluSerA

1351  GGGACCTCCTTATAGATGAGTGGAAAAGCCTGACCTATGATGAAGTCATCAGCTTTGTGCCACCACCCCTTGACC
 331  rgAspLeuLeuIleAspGluTrpLysSerLeuThrTyrAspGluValIleSerPheValProProProLeuAspG

1426  AAGAAGAGATGGAGTCCTGAGCACCTGGTTTCTGTTCTGTTGATCCCACTTCACTGTGAGGGGAAGGCCTTTTCA
 356  lnGluGluMetGluSerEnd

1501  CGGGAACTCTCCAAATATTATTCAAGTGCCTCTTGTTGCAGAGATTTCCTCCATGGTGGAAGGGGGTGTGCGTGC

1576  GTGTGCGTGCGTGTTAGTGTGTGTGCATGTGTGTGTCTGTCTTTGTGGGAGGGTAAGACAATATGAACAAACTAT

1651  GATCACAGTGACTTTACAGGAGGTTGTGGATGCTCCAGGGCAGCCTCCACCTTGCTCTTCTTTCTGAGAGTTGGC

1726  TCAGGCAGACAAGAGCTGCTGTCCTTTTAGGAATATGTTCAATGCAAAGTAAAAAAATATGAATTGTCCCCAATC
```

FIG.2IC

```
1801  CCGGTCATGCTTTTGCCACTTTGGCTTCTCCTGTGACCCCACCTTGACGGTGGGGCGTAGACTTGACAACATCCC
1876  ACAGTGGCACGGAGAGAAGGCCCATACCTTCTGGTTGCTTCAGACCTGACACCGTCCCTCAGTGATACGTACAGC
1951  CAAAAAGGACCAACTGGCTTCTGTGCACTAGCCTGTGATTAACTTGCTTAGTATGGTTCTCAGATCTTGACAGTA
2026  TATTTGAAACTGTAAATATGTTTGTGCCTTAAAAGGAGAGAAGAAAGTGTAGATAGTTAAAAGACTGCAGCTGCT
2101  GAAGTTCTGAGCCGGGCAAGTCGAGAGGCTGTTGGACAGCTGCTTGTGGGCCCGGAGTAATCAGGCAGCCTTCA
2176  TAGGCGGTCATGTGTGCATGTGAGCACATGCGTATATGTGCGTCTCTCTTTCTCCCTCACCCCCAGGTGTTGCCA
2251  TTTCTCTGCTTACCCTTCACCTTTGGTGCAGAGGTTTCTTGAATATCTGCCCCAGTAGTCAGAAGCAGGTTCTTG
2326  ATGTCATGTACTTCCTGTGTACTCTTTATTTCTAGCAGAGTGAGGATGTGTTTTGCACGTCTTGCTATTTGAGCA
2401  TGCACAGCTGCTTGTCCTGCTCTCTTCAGGAGGCCCTGGTGTCAGGCAGGTTTGCCAGTGAAGACTTCTTGGGTA
2476  GTTTAGATCCCATGTCACCTCAGCTGATATTATGGCAAGTGATATCACCTCTCTTCAGCCCCTAGTGCTATTCTG
2551  TGTTGAACACAATTGATACTTCAGGTGCTTTTGATGTGAAAATCATGAAAAGAGGAACAGGTGGATGTATAGCAT
2626  TTTTATTCATGCCATCTGTTTTCAACCAACTATTTTTGAGGAATTATCATGGGAAAAGACCAGGGCTTTTCCCAG
2701  GAATATCCCAAACTTCGGAAACAAGTTATTCTCTTCACTCCCAATAACTAATGCTAAGAAATGCTGAAAATCaAA
```

FIG.21D

2776 GTAAAAAATTAAAGCCCATAAGGCCAGAAACTCCTTTTGCTGTCTTTCTCTAAATATGATTACTTTAAAATAAAA

2851 AAGTAACAAGGTGTCTTTTCCACTCCTATGGAAAAGGGTCTTCTTGGCAGCTTAACATTGACTTCTTGGTTTGGG

2926 GAGAAATAAATTTTGTTTCAGAATTTTGTATATTGTAGGAATCCCTTTGAGAATGTGATTCCTTTTGATGGGGAG

3001 AAAGGGCAAATTATTTTAATATTTTGTATTTTCAACTTTATAAAGATAAAATATCCTCAGGGGTGGAGAAGTGTC

3076 GTTTTCATAACTTGCTGAATTTCAGGCATTTTGTTCTACATGAGGACTCATATATTTAAGCCTTTTGTGTAATAA

3151 GAAAGTATAAAGTCACTTCCAGTGTTGGCTGTGTGACAGAATCTTGTATTTGGGCCAAGGTGTTTCCATTTCTCA

3226 ATCAGTGCAGTGATACATGTACTCCAGAGGGACRGGGTGGACCCCCTGAGTCAACTGGAGCAAGAAGGAAGGAGG

3301 CAGACTGATGGCGATTCCCTCTCACCCGGGACTCTCCCCCTTTCAAGGAAAGTGAACCTTTAAAGTAAAGGCCTC

3376 ATCTCCTTTATTGCAGTTCAAATCCTCACCATCCACAGCAAGATGAATTTTATCAGCCATGTTTGGTTGTAAATG

3451 CTCGTGTGATTTCCTACAGAAATACTGCTCTGAATATTTTGTAATAAAGGTCTTTGCACATGTGACCACATACGT

3526 GTTAGGAGGCTGCATGCTCTGGAAGCCTGGACTCTAAGCTGGAGCTCTTGGAAGAGCTCTTCGGTTTCTGAGCAT

3601 AATGCTCCCATCTCCTGATTTCTCTGAACAGAAAACAAAAGAGAGAATGAGGGAAATTGCTATTTTATTTGTATT

3676 CATGAACTTGGCTGTAATCAGTTATGCCGTATAGGATGTCAGACAATACCACTGGTTAAAATAAAGCCTATTTTT

3751 CAAATTTAAAAAAAAAAAAAAAAAA 3775

FIG.2IE

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   1  CGCCCCAGTCGCAGGGGCACATCCAGCCGCTGCGGCTGACAGCAGCCGCGCGCGCGGGAGTCTGCGGGGTCGCGG

  76  CAGCCGCACCTGCGCGGGCGACCAGCGCAAGGTCCCCGCCCGGCTGGGCGGGCAGCAAGGGCCGGGGAGAGGGTG

 151  CGGGTGCAGGCGGGGGCCCCACAGGGCCACCTTCTTGCCCGGCGGCTGCCGCTGGAAAATGTCTCAGGAGAGGCC
 -18                                                                MetSerGlnGluArgPr

 226  CACGTTCTACCGGCAGGAGCTGAACAAGACAATCTGGGAGGTGCCCGAGCGTTACCAGAACCTGTCTCCAGTGGG
   7  oThrPheTyrArgGlnGluLeuAsnLysThrIleTrpGluValProGluArgTyrGlnAsnLeuSerProValGl

 301  CTCTGGCGCCTATGGCTCTGTGTGTGCTGCTTTTGACACAAAAACGGGGTTACGTGTGGCAGTGAAGAAGCTCTC
  32  ySerGlyAlaTyrGlySerValCysAlaAlaPheAspThrLysThrGlyLeuArgValAlaValLysLysLeuSe

 376  CAGACCATTTCAGTCCATCATTCATGCGAAAAGAACCTACAGAGAACTGCGGTTACTTAAACATATGAAACATGA
  57  rArgProPheGlnSerIleIleHisAlaLysArgThrTyrArgGluLeuArgLeuLeuLysHisMetLysHisGl

 451  AAATGTGATTGGTCTGTTGGACGTTTTTACACCTGCAAGGTCTCTGGAGGAATTCAATGATGTGTATCTGGTGAC
  82  uAsnValIleGlyLeuLeuAspValPheThrProAlaArgSerLeuGluGluPheAsnAspValTyrLeuValTh

 526  CCATCTCATGGGGGCAGATCTGAACAACATTGTGAAATGTCAGAAGCTTACAGATGACCATGTTCAGTTCCTTAT
 107  rHisLeuMetGlyAlaAspLeuAsnAsnIleValLysCysGlnLysLeuThrAspAspHisValGlnPheLeuIl

 601  CTACCAAATTCTCCGAGGTCTAAAGTATATACATTCAGCTGACATAATTCACAGGGACCTAAAACCTAGTAATCT
 132  eTyrGlnIleLeuArgGlyLeuLysTyrIleHisSerAlaAspIleIleHisArgAspLeuLysProSerAsnL

 676  AGCTGTGAATGAAGACTGTGAGCTGAAGATTCTGGATTTTGGACTGGCTCGGCACACAGATGATGAAATGACAGG
 157  uAlaValAsnGluAspCysGluLeuLysIleLeuAspPheGlyLeuAlaArgHisThrAspAspGluMetThrGl
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FIG.22A

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751   CTACGTGGCCACTAGGTGGTACAGGGCTCCTGAGATCATGCTGAACTGGATGCATTACAACCAGACAGTTGATAT
182   yTyrValAlaThrArgTrpTyrArgAlaProGluIleMetLeuAsnTrpMetHisTyrAsnGlnThrValAspIl

826   TTGGTCAGTGGGATGCATAATGGCCGAGCTGTTGACTGGAAGAACATTGTTTCCTGGTACAGACCATATTGATCA
207   eTrpSerValGlyCysIleMetAlaGluLeuLeuThrGlyArgThrLeuPheProGlyThrAspHisIleAspGl

901   GTTGAAGCTCATTTTAAGACTCGTTGGAACCCCAGGGGCTGAGCTTTTGAAGAAAATCTCCTCAGAGTCTGCAAG
232   nLeuLysLeuIleLeuArgLeuValGlyThrProGlyAlaGluLeuLeuLysLysIleSerSerGluSerAlaAr

976   AAACTATATTCAGTCTTTGACTCAGATGCCGAAGATGAACTTTGCGAATGTATTTATTGGTGCCAATCCCCTGGC
257   gAsnTyrIleGlnSerLeuThrGlnMetProLysMetAsnPheAlaAsnValPheIleGlyAlaAsnProLeuAl

1051  TGTCGACTTGCTGGAGAAGATGCTTGTATTGGACTCAGATAAGAGAATTACAGCGGCCCAAGCCCTTGCACATGC
282   aValAspLeuLeuGluLysMetLeuValLeuAspSerAspLysArgIleThrAlaAlaGlnAlaLeuAlaHisAl

1126  CTACTTTGCTCAGTACCACGATCCTGATGATGAACCAGTGGCCGATCCTTATGATCAGTCCtTTGAAAGCAGGGA
307   aTyrPheAlaGlnTyrHisAspProAspAspGluProValAlaAspProTyrAspGlnSerPheGluSerArgAs

1201  CCTCCTTATAGATGAGTGGAAAAGCCTGACCTATGATGAAGTCATCAGCTTTGTGCCACCACCCCTTGACCAAGA
332   pLeuLeuIleAspGluTrpLysSerLeuThrTyrAspGluValIleSerPheValProProProLeuAspGlnGl

1276  AGAGATGGAGTCCTGAGCACCTGGTTTCTGTTCTGTTGATCCCACTTCACTGTGAGGGGAAGGCCTTTTCACGGG
357   uGluMetGluSerEnd

1351  AACTCTCCAAATATTATTCAAGTGCCAAAAA  1381
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FIG.22B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10529

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/00, 21/04; C07K 14/435; C12N 15/00; C12Q 1/00
US CL :536/23.5; 530/350; 435/320.1, 252.3, 69.1, 7.2; 436/503, 504
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 530/350; 435/320.1, 252.3, 69.1, 7.2; 436/503, 504

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG - BIOTECH FILES, GENEMBL SEQUENCE DATABASES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US, A, 5,317,019 (BENDER ET AL.) 31 May 1994, see entire document.	1-20
A	US, A, 4,794,114 (BENDER ET AL.) 27 December 1988, see entire document.	1-20
A	US, A, 4,780,470 (BENDER ET AL.) 25 October 1988, see entire document.	1-20
A	US, A, 4,778,806 (BENDER ET AL.) 18 October 1988, see entire document.	1-20
A	Agents and Actions, Volume 27, No. 3/4, issued 1989, J. C. Lee et al, "Effect of SK & F 86002 on cytokine production by human monocytes", pages 277-279, see entire document.	1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 DECEMBER 1994	Date of mailing of the international search report 09 JAN 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ELIZABETH C. KEMMERER Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

BOX II. OBSERVATIONS WHERE UNITY F INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-20, drawn to nucleic acids encoding a CSBP, the protein, vectors, host cells, a method of expressing the nucleic acids, and a method of identifying a CSAID using the protein.

II. Claims 21-23, drawn to a method of identifying CSBP ligands.

III. Claims 24 and 25, drawn to antagonists and agonists of CSBP, and pharmaceutical compositions comprising same.

IV. Claim 26, drawn to antisense molecules.

V. Claims 27 and 28, drawn to antibodies.

VI. Claim 29, drawn to a transgenic animal.

VII. Claim 30, drawn to a method of screening compounds using a fusion protein.

The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept. Beginning with Invention II, each subsequent invention introduces a new concept not required by Invention I or any of the other Inventions. For example, Invention II introduces the concept of ligands and natural sources thereof, Invention III introduces antagonists/antagonists and chemical sources thereof, Invention IV introduces antisense technology which is useful in gene expression modulation and nucleic acid probing techniques, Invention V introduces antibodies and the entire field of immunology, Invention VI introduces transgenic technology, and Invention VII introduces the field of fusion protein engineering.